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14. ABSTRACT
The primary objective of this application is to establish how Fibulin-5 enhances the oncogenic activities of TGF-beta, particularly its ability to stimulate breast cancer invasion and metastasis. We hypothesized that inactivating Fibulin-5 function will prevent the conversion of TGF-beta from a suppressor to a promoter of breast cancer growth and invasion, thereby alleviating breast cancer development and progression stimulated by TGF-beta. Major findings of the past funding cycle include the ability of Fibulin-5 to (i) bind integrins on mammary epithelial cells (MECs) independent of its integrin-binding RGD motif; (ii) interact physically with TGF-beta and enhance its presentation to TGF-beta receptors; (iii) promote epithelial-mesenchymal transition in an integrin-independent manner in part by stimulating the expression of Cox-2, PAI-1, and MMP-9; (iv) enhance MEC proliferation by activating FAK and ERK1/2; and (v) induce MEC resistance to apoptosis and anoikis by stimulating NF-kappaB activation, by inducing survivin and xIAP expression, and by repressing TNF-alpha expression. Finally, we determined that Fibulin-5 expression is greatly augmented during breast cancer progression, particularly at the point when malignant MECs acquire metastatic phenotypes. This important finding implicates Fibulin-5 as a potential marker for breast cancer metastasis and reinforces the need to target Fibulin-5 chemotherapeutically in patients with metastatic disease.

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INTRODUCTION:

Breast cancer is the second leading cause of cancer death in women in the United States. Invasion and metastasis are the most lethal characteristics of breast cancer and the leading cause of breast cancer-related death. TGF-β normally inhibits breast cancer development by preventing mammary epithelial cell (MEC) proliferation, or by inducing MEC apoptosis. Mammary tumorigenesis counteracts the tumor suppressing activities of TGF-β, thus enabling TGF-β to stimulate breast cancer invasion and metastasis. Fundamental gaps exist in our knowledge of how malignant MECs overcome the cytostatic actions of TGF-β, and of how TGF-β stimulates the development and progression of mammary tumors. These knowledge gaps have prevented science and medicine from implementing treatments effective in antagonizing the oncogenic activities of TGF-β in developing and progressing breast cancers. We recently discovered that the expression and activity of the TGF-β gene target, Fibulin-5 (FBLN5), potentiates TGF-β stimulation of invasion and epithelial-mesenchymal transition (EMT) in normal and malignant MECs *in vitro*, and more importantly, enhances the growth and pulmonary metastasis of mammary tumors in mice. Interestingly, we find that FBLN5 incorporates into active TGF-β receptor complexes in a β3 integrin-dependent manner, an event associated with the activation of intracellular signaling by TGF-β. Based on these and other compelling findings, we hypothesized that inactivating FBLN5 function will prevent the conversion of TGF-β from a suppressor to a promoter of breast cancer growth and invasion, thereby alleviating breast cancer development and progression stimulated by TGF-β. The goals of this project are to determine the molecular mechanisms that mediate incorporation of FBLN5 into active TGF-β receptor complexes, and to determine the role of FBLN5 in mediating β3 integrin and Src activation, leading to oncogenic signaling by TGF-β in normal and malignant MECs. Finally, we will determine whether interdicting FBLN5 function abrogates the oncogenic activities of TGF-β and prevents its stimulation of breast cancer progression *in vivo*. These studies are important because they will *(i)* provide valuable information on how breast cancers develop and progress, and on how TGF-β promotes these processes; *(ii)* identify the signaling mechanisms and systems that mediate the oncogenic nature of TGF-β; and *(iii)* identify FBLN5 antagonists capable of alleviating the oncogenic activities of TGF-β, as well as establish their effectiveness in preventing breast cancer progression stimulated by TGF-β. Moreover, application of our findings will enable science and medicine to one day improve the prognosis and treatment of patients with metastatic breast cancer

BODY:

Overview and General Findings: The specific aims of the proposed research have not been modified. Indeed, our recently published manuscript in the journal *Carcinogenesis* [1] clearly established the importance of FBLN5 in promoting epithelial-mesenchymal transition (EMT) in normal and malignant mammary epithelial cells (MECS). Equally important, we showed that FBLN5 expression greatly enhanced the ability of TGF-β to stimulate EMT, as well as promoted its oncogenic activities in normal and malignant MECs both *in vitro* and *in vivo*. Clearly, elucidating the molecular mechanisms that enable FBLN5 to enhance oncogenic TGF-β signaling has tremendous potential to neutralize the metastasis promoting activities of this multifunctional cytokine, and as such, to ultimately improve the clinical course of breast cancer patients with metastatic disease.

Data in the scientific literature has recently established the essential role of TGF-β in regulating the activities of breast cancer-associated fibroblasts and stromal components [2-5]. Indeed, mounting evidence indicates that TGF-β promotes breast cancer progression in part *via* its reprogramming of MEC microenvironments and their cellular architectures. Moreover, TGF-β also induces desmoplastic and fibrotic reactions that elicit the formation of tense, rigid tumor microenvironments that *(i)* enhance the selection and expansion of developing mammary neoplasms, particularly that of late-stage

metastatic cells, and *(ii)* predict for poor clinical outcomes in breast cancer patients. Our previous published studies established FBLN5 as an important stromal-produced secreted factor that regulates tumor development in mice [6-9]. Thus, we characterized changes in the fibroblast transcriptome elicited by FBLN5, or by FBLN5 plus TGF-β. Microarray analyses identified 1181 genes whose expression is regulated by FBLN5, and an additional 1675 genes whose expression is regulated by TGF-β. Differential expression of 14 individual genes was verified by semi-quantitative real-time PCR. Downregulated FBLN5 gene targets included *a)* BB503935; *b)* pleckstrin-homology domain-containing family A member; *c)* transglutaminase-2; and *d)* Rho GTPase activating protein 24. Upregulated FBLN5 gene targets included *a)* BB533736; *b)* BB831146; *c)* HoxD9; *d)* thrombospondin-1; *e)* collagen type XI; *f)* angiopoietin-1; *g)* cysteine-rich protein 61; *h)* Dkk3; *i)* fibromodulin; and *j)* HoxD10. Oncomine analyses showed the expression of fibromodulin to be upregulated in human breast cancers, and as such, we further characterized the activities of this novel FBLN5 gene target. In doing so, we found that fibromodulin expression greatly enhanced the coupling of TGF-β to Smad2/3 and AP-1 activation, while simultaneously abrogating both basal and TGF-β-stimulated NF-κB activation in fibroblasts. Importantly, we observed fibromodulin expression to stabilize that of the NF-κB inhibitory protein, IκBα. We further determined that fibromodulin stabilized IκBα expression by activating JNK and CK-II, which inactivate calpain and its proteolytic activity against IκBα. Thus, in addition to inhibiting NF-κB activity in fibroblasts, the activation of this fibromodulin-dependent pathway promotes apoptosis in fibroblasts. Our findings related to this novel FBLN5:fibromodulin signaling axis are now being prepared for publication. **Clinically, chemotherapeutic targeting of this pathway may offer novel inroads into alleviating the oncogenic activities of TGF-β in breast cancer stroma.**

Based on our findings presented below, we remain convinced that our analyses of noncanonical and oncogenic effectors targeted by FBLN5 and TGF-β will enable the development of safer, more directed chemotherapies capable of phenotypically normalizing and reverting the malignant behaviors of developing and progressing breast cancers.

Task-Specific Findings:

Task 1: ***Determine the molecular mechanisms that mediate incorporation of FBLN5 into active TGF-β receptor complexes.*** We previously engineered normal NMuMG and metastatic 4T1 cells to stably express β3 integrin or its inactive mutant, D119A-β3 integrin [10-12]. Our previously published studies demonstrated the function of β3 integrin in promoting oncogenic TGF-β signaling, including its ability to stimulate EMT and pulmonary metastasis of breast cancer cells [10-12]. We recently introduced wild-type FBLN5 and its RGE-mutant, which we demonstrated previously to prevent FBLN5 from ligating integrins on endothelial cells [7]. The functional characteristics of these FBLN5 and β3 integrin manipulations on MEC behavior in response to TGF-β are quite interesting and will be discussed below (*see Task 2).* With respect to the primary objective of Task 1 – *i.e.,* to identify the molecular determinants that mediate incorporation of FBLN5 into active TGF-β receptor complexes, and more importantly, to determine the impact of disrupting the formation of these complexes on normal and malignant MEC response to TGF-β – our preliminary data indicate that FBLN5 is capable of binding β3 integrin on MECs independent of its integrin-binding RGD motif. Indeed, we find that MEFs derived from FBLN5-deficient embryos respond poorly to TGF-β, and that re-expression of either wild-type FBLN5 or RGE-FBLN5 molecules in these FBLN5-deficient MEFs significantly enhance MEF response to TGF-β. Thus, our findings to date suggest that FBLN5 may incorporate into TGF-β receptor complexes independent of traditional integrin-binding activities. Alternatively, FBLN5 may incorporate into TGF-β receptor complexes in a manner wholly independent of β3 integrin. With respect to the former possibility, we now are optimizing the expression and purification systems necessary to isolate various recombinant FBLN5 mutants, including full-length wild-type and RGE-

mutant FBLN5 molecules, as well as those mutants that lack the N-terminal Pro-rich domain (*i.e.,* ΔPro), the entire N-terminal domain (*i.e.,* ΔNT), and the entire globular C-terminal domain (*i.e.,* ΔCT) or those that only contain the N-terminal (*i.e.,* NT-FBLN5) or C-terminal (*i.e.,* CT-FBLN5). FBLN5 mutants found to incorporate into TGF-β receptor complexes will then be subjected to gross- and fine-deletion analyses, followed by Ala-scanning mutagenesis to elucidate the molecular determinants that mediate FBLN5 association with TGF-β receptors. We fully expect to possess engineered FBLN5 molecules that are incapable of supporting oncogenic TGF-β signaling by the completion of Year 2, and to complete a thorough characterization of their impact on TGF-β signaling and breast cancer cell behavior during Year 3.

Because our initial studies of FBLN5 incorporation into TGF-β receptor complexes showed that wild-type and RGE-FBLN5 were both capable of capturing β3 integrin in immunocomplex assays, we began to consider the possibility that FBLN5 may incorporate into TGF-β receptors in an integrin-independent fashion. In support of this notion, we found that FBLN5 bears striking homology to members of LTBP (latent TGF-β-binding proteins) family of proteins, particularly in their calcium-binding EGF-like repeats. Thus, we hypothesized that FBLN5 may bind directly to TGF-β, which then pulls FBLN5 into TGF-β receptor complexes. Accordingly and quite surprisingly, we used three separate and distinct binding protocols to show unambiguously that FBLN5 does indeed interact physically with active TGF-β independent of whether FBLN5 can bind to integrins (*i.e.,* wild-type FBLN5 and RGE-FBLN5 bind indistinguishably to active TGF-β). **This finding represents a major advance for TGF-β and FBLN5 biologists, and may in fact explain why FBLN5-deficient MEFs are unresponsive to TGF-β**. Indeed, our findings indicate that FBLN5 may function in binding directly to TGF-β and facilitating its presentation and/or incorporation to inactive TGF-β receptor complexes, resulting in enhanced transmembrane signaling initiated by TGF-β. Accordingly, MECs engineered to overexpress FBLN5 exhibit significantly elevated levels of Smad2/3 activity as compared to their GFP-expressing counterparts, a finding consistent with FBLN5 functioning to present and enhance autocrine TGF-β signaling in normal and malignant MECs. We have now engineered MECs to produce various FBLN5 mutants to map the domains operant in mediate its interaction with TGF-β. After affirming which regions of FBLN5 bind TGF-β1, we will immediately generate FBLN5 mutants that lack this domain/motif to assess how preventing FBLN5 from binding TGF-β impacts normal and malignant MEC response to TGF-β both *in vitro* and *in vivo.* As above, we fully expect to complete this exciting and important task during Year 2, and to complete a thorough characterization of their impact on TGF-β signaling and breast cancer cell behavior during Year 3

Task 2: ***Determine the role of FBLN5 in mediating β3 integrin and Src activation, leading to oncogenic signaling by TGF-β in normal and malignant MECs.*** The primary objective of Task 2 is to identify FBLN5 effectors operant in mediating oncogenic signaling by TGF-β. In this regard, we have found that wild-type and RGE-FBLN5 are both capable of promoting partial EMT phenotype in normal MECs. Interestingly, we also observed the combination of FBLN5 and β3 integrin to significantly enhance the proliferative potential of normal MECs, a response that was not recapitulated in MECs co-expressing RGE-FBLN5 and β3 integrin. In addition, the combined expression of FBLN5 and β3 integrin greatly attenuated the sensitivity of MECs to the cytostatic activities of TGF-β. The enhanced response of MECs to FBLN5 also correlated with its ability to significantly augment the activation of FAK and ERK1/2 in these same cells. Thus, FBLN5 expression induced by TGF-β in normal and malignant MECs appears to play a significant role in mediating its growth promoting activities in MECs. Along these lines and in stark contrast to its effects in fibroblasts [9], we find that FBLN5 greatly enhances basal and TGF-β-stimulated NF-κB activity in normal and malignant MECs in part *via* promoting increased degradation of IκBα. Indeed, we recently found that EMT induced by

TGF-β initiates a pro-survival gene expression profile, such that MECs that survive the EMT process are more resistant to apoptosis and anoikis. Given our published work that FBLN5 promotes EMT in normal and malignant MECs [1], we reasoned that FBLN5 expression would also promote survival signaling in these same cells. Accordingly, we now find that FBLN5 greatly suppresses TNF-α expression (by 90%) in normal MECs, while simultaneously stimulating that of the *(i)* survival factors, survivin and xIAP; *(ii)* angiogenic and EMT molecule, Cox-2; *(iii)* pro-metastatic molecule, PAI-1; and *(iv)* pro-invasion and EMT-molecule, MMP-9. **These findings are a major advance to the fibulin field, and we now are rapidly extending these findings to the aforementioned normal and malignant MECs engineered to express all combinations of wild-type and mutant FBLN5 and β3 integrin molecules**. In addition, we have begun manipulating the expression of these FBLN5 gene targets in normal and malignant MECs to access their role in regulating MEC response to TGF-β both *in vitro* and *in vivo.*

Task 3: ***Determine whether interdicting FBLN5 function abrogates the oncogenic activities of TGF-β and prevents its stimulation of breast cancer progression in vivo***. The primary objective of Task 3 is to establish the effectiveness of abolishing FBLN5 function and its subsequent incorporation into active TGF-β receptor complexes to prevent breast cancer progression and metastasis induced by TGF-β. As mentioned above, this past year saw us identify a variety of novel FBLN5 gene targets, as well as uncover two potentially important tumor promoting functions for FBLN5, namely its ability to facilitate the presentation of TGF-β to its receptors and its potential to induce survival signaling in normal and malignant MECs. In the next year, we will rapidly test these FBLN5 functions using malignant, nonmetastatic 67NR and malignant, highly metastatic 4T1 cells that will be engineered to stably express FBLN5 mutants that fail to bind and present TGF-β to its receptors, as well as those construct derivatives of these breast cancer cell lines whose expression of FBLN5 target genes has been positively and negatively manipulated. Afterward, the impact of these manipulations on primary tumor growth and metastasis will be assessed in syngeneic Balb/C mice.

KEY RESEARCH ACCOMPLISHMENTS:

- Mammary tumorigenesis upregulates FBLN5 expression, particularly at the point when breast cancer cell acquire metastatic phenotypes
- A novel FBLN5 gene signature has now been identified and established
- The FBLN5 gene target, fibromodulin, suppresses NF-κB activity by stabilizing IκBα expression
- Stabilization of IκBα transpires via JNK and CK-II activation, which conspire to inactivate calpain and its proteolytic activity against IκBα
- Activation of this fibromodulin signaling axis promotes apoptosis
- FBLN5 interacts with β3 integrin in an RGD-independent fashion
- FBLN5 binds TGF-β, leading to its enhanced presentation to TGF-β receptors and elevated autocrine TGF-β signaling in normal and malignant MECs
- FBLN5 and β3 integrin promote normal and malignant MEC proliferation, a cellular response coupled to FAK and ERK1/2 activation by FBLN5
- RGE-FBLN5 and β3 integrin fail to induce MEC proliferation
- FBLN5 induces survival signaling in normal and malignant MECs in part by strongly activating NF-κB
- Survival signaling by FBLN5 is also coupled to its ability to suppress TNF-α expression, and to induce that of survivin and xIAP
- FBLN5 potentially induces breast cancer cell EMT, migration, and invasion by upregulating the expression of Cox-2, PAI-1, and MMP-9

REPORTABLE OUTCOMES:

Schiemann Laboratory Publications Acknowledging Support of BC084651:

Keshamouni, V.G. and **Schiemann, W.P.** (2009) EMT in Tumor Metastasis: A Method to the Madness. **Future Oncology** *5,* 1109-1111.

Wendt, M.K., Allington, T.M. and **Schiemann, W.P.** (2009) Mechanisms of epithelial-mesenchymal transition by TGF-β in normal and malignant cells. **Future Oncology** *5,* 1145-1168.

Tian, M. and **Schiemann, W.P.** (2009) The TGF-β paradox in human cancer: An update. **Future Oncology** *5,* 259-271.

Wendt, M.K., Smith, J.A. and **Schiemann, W.P.** (2009) p130Cas is required for mammary tumor growth and TGF-β-mediated metastasis through regulation of Smad2/3 activity. **Journal of Biological Chemistry** *284, In Press. (*PMID: 19822523)

Schiemann Laboratory Meeting and Invited Seminar Presentations Acknowledging Support of BC084651:

Schiemann, W.P. (2009) Oncogenic TGF-β signaling in breast cancer. **UC-Davis Cancer Center,** Sacramento, CA. (May 14, 2009).

Schiemann, W.P. (2009) Oncogenic TGF-β signaling in breast cancer. **Case Comprehensive Cancer Center,** Cleveland, OH. (July 16, 2009).

Schiemann, W.P. (2009) Activated Abl kinase inhibits oncogenic TGF-β signaling, EMT, and tumorigenesis in mammary tumors. **The EMT International Association's 4th International Meeting on "Epithelial-Mesenchymal Transition,"** Tucson, AZ. (September 23, 2009).

Schiemann, W.P. (2009) The Abl and Cain of TGF-β signaling. **Department of Pharmacology,** Case Western Reserve University, Cleveland, OH. (October 5, 2009).

CONCLUSION:

Our findings have clearly established new biological and pathological paradigms for FBLN5 and TGF-β. Importantly, we continue to *(i)* elucidate the mechanisms whereby FBLN5 induces oncogenic TGF-β signaling in normal and malignant MECs, and *(ii)* identify the FBLN5 effectors that contribute to the invasive and metastatic properties of TGF-β. Equally importantly, our findings have provided the first FBLN5 gene signature that underlies its biological activities, and this dataset has already uncovered fibromodulin as a novel FBLN5 gene target that regulates fibroblast survival. Our findings that FBLN5-deficient MEFs are largely unresponsive to TGF-β is exciting and may in fact be explained by our demonstration that FBLN5 binds directly to TGF-β, leading to its presentation to TGF-β receptors and the enhanced activation of autocrine TGF-β signaling in normal and malignant MECs. Our findings have also identified several novel FBLN5 effectors whose activity contributes to oncogenic TGF-β signaling. Given our recent finding that developing and progressing mammary tumors significantly upregulate their expression of FBLN5 at the point at which these tumors become metastatic, our results clearly establish FBLN5 as a new and potentially important biomarker to detect

and track metastatic disease in patients with breast cancer. Moreover, the ability of FBLN5-deficiency to significantly attenuate cellular responses to TGF-β suggest that measures capable of antagonizing FBLN5 function may alleviate the initiation of oncogenic TGF-β signaling. Indeed, successful identification and implementation of FBLN5 molecules that are unable to bind and present TGF-β to its receptors on metastatic breast cancer cells holds tremendous potential to alleviate metastatic disease in breast cancer patients. Thus, translation of our findings will provide a novel set of biomarkers comprised of FBLN5 and its effectors that will be capable of predicting whether or not malignant MECs possess metastatic phenotypes. In addition, our findings will offer new inroads to target these metastatic lesions via employment of FBLN5 mutants that will suppress oncogenic TGF-β signaling in breast cancer cells. Collectively, we envision that further developing these reagents and clinical protocols will play a significant role in developing a "personalized medicine" approach tailored to treat individuals with metastatic breast cancer.

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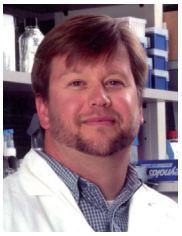
APPENDICES:

Contains Schiemann Laboratory Publications Acknowledging Support of BC084651 as Listed Under "REPORTABLE OUTCOMES"

Epithelial–mesenchymal transition in tumor metastasis: a method to the madness



"Dissemination of cancer is not simply a random dispersion of cells, but instead represents an ordered and systematic method to this madness."

Venkateshwar G Keshamouni† & William P Schiemann

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Were it not for the ability of carcinoma cells to metastasize and colonize distant organs, all solid tumors would present medically as a group of chronic but manageable diseases. There has been significant progress in the understanding of how cancer cells acquire five of the six essential hallmarks proposed for their transformation [1]. Unfortunately, it still remains unclear as to how and when cancer cells acquire the ability to metastasize – that is, the sixth and final hallmark that is responsible for more than 90% of cancer-related mortality [1]. However, it has long been recognized that the dissemination of cancer is not simply a random dispersion of cells, but instead represents an ordered and systematic method to this madness. Indeed, epithelial–mesenchymal transition (EMT) is one such method that has been proposed to initiate the metastasis of carcinoma cells [2].

Epithelial–mesenchymal transition was first recognized as a conserved embryonic and developmental process that facilitates the dispersion of cells that ultimately leads to the generation of distinct tissue types [3]. In undergoing EMT, cells lose their epithelial properties, while acquiring mesenchymal properties that enable transitioned cells to migrate to predetermined destinations [4]. The idea that a similar process is reactivated during tumor progression and other pathologies, including wound healing, tissue regeneration and organ fibrosis, has gained significant ground and acceptance in recent years. Indeed, this fact is readily apparent in the sheer number of publications on this topic, and in the number of EMT-focused sessions and dedicated meetings that have grown exponentially in the last few years. It is now widely accepted that EMT plays an important role during tumor progression and confers certain fundamental abilities to cancer cells that are essential for tumor metastasis. These include the ability to migrate, resist anoikis and induce immunosuppression [5–7].

The precise contribution of EMT to tumor metastasis is still a subject of considerable debate in the scientific literature [8]. Recent reports of EMT in *in vivo* animal models and human studies [9–12], to a certain degree, have softened the arguments for lack of concrete *in vivo* evidence. However, convincing demonstration of a true phenotypic switch is still yet to come. The other dismissive argument that EMT is simply reflective of genomic instability in cancer cells is also fading in light of increasing numbers of studies reporting EMT that occurs in normal epithelial cells from various organs in response to injury [9,11,13].

Reports of EMT conferring resistance to certain classes of drugs and therapeutic modalities, and correlation of EMT gene signatures with poor outcomes have been described [14–16]. These observations, together with the recent finding that EMT may confer stem cell-like properties to resulting mesenchymal cells [17] have highlighted the clinical relevance of this process. Consequently, several groups, both in industry and academia, are actively pursuing the discovery of novel molecules to target EMT [18].

"...any effort to identify context-specific signals should consider the physiological state of the epithelium in which EMT is taking place – that is, whether it transpires in normal, transformed or injured epithelium..."

Recently, Kalluri and Weinberg proposed to classify EMT into three distinct subtypes based on the biological context in which they

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occur [4]. This new terminology was not available at the time the reviews for this special focus issue were accepted for publication, and as such, this classification is not used herein. With the exception of the review by Micalizzi *et al.* [19], the other articles have predominantly discussed what now could be referred to as type III EMT in the new classification system, which is EMT in the context of tumor progression. By contrast, the article by Micalizzi *et al.* describes the regulators of developmental EMT, which now is known as type I EMT in the new classification scheme, and discusses the transcriptional reactivation of type I EMT in the context of type III EMT. Particularly interesting is the discussion of their own work investigating the role of two new players, Six1 and Six4, in the EMT of mouse mammary tumors. Radaelli *et al.* provide a very elegant historical perspective by discussing some of the early descriptions of EMT in mouse tumors [20], some of which date as far back as the year 1854. They also present an interesting comparison of EMT in mouse and human pathologies. A very comprehensive review of the regulatory pathways implicated in TGF-β-induced EMT in normal and malignant cells of the breast is provided in the article by Wendt *et al.* [21], and finally, van Zijl *et al.* [22] review the evidence for EMT in hepatocellular carcinoma and discussed its implications for the treatment of these tumors.

> "Given the dramatic changes that take place during EMT, it is wholly reasonable to expect EMT to also elicit powerful alterations within tumor microenvironments, as well as to target the activities and behaviors of various stromal supporting cells."

Pathways and molecules that distinguish EMT in tumor progression from the other two biological contexts are far from clear. However, any effort to identify context-specific signals should consider the physiological state of the epithelium in which EMT is taking place – that is, whether it transpires in normal, transformed or injured epithelium, and how these unique epithelial states impact the functional consequences of the resulting EMT. Indeed, the vast majority of EMT studies to date have solely focused on assessing the functional consequences of EMT in solely altering the behaviors and functions of tumor cells, not their accompanying stromal components. Given the dramatic changes that take place during EMT, it is wholly reasonable to expect EMT to also elicit powerful alterations within tumor microenvironments, as well as to target the activities and behaviors of various stromal supporting cells. Therefore, the implications of EMT on the interactions of tumor cells with their accompanying stromal and microenvironmental components clearly need to be explored in future studies.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Bibliography

Papers of special note have been highlighted as:
▪ of interest
▪▪ of considerable interest

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Review
Future Oncology

Mechanisms of the epithelial–mesenchymal transition by TGF-β

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The formation of epithelial cell barriers results from the defined spatiotemporal differentiation of stem cells into a specialized and polarized epithelium, a process termed mesenchymal–epithelial transition. The reverse process, epithelial–mesenchymal transition (EMT), is a metastable process that enables polarized epithelial cells to acquire a motile fibroblastoid phenotype. Physiological EMT also plays an essential role in promoting tissue healing, remodeling or repair in response to a variety of pathological insults. On the other hand, pathophysiological EMT is a critical step in mediating the acquisition of metastatic phenotypes by localized carcinomas. Although metastasis clearly is the most lethal aspect of cancer, our knowledge of the molecular events that govern its development, including those underlying EMT, remain relatively undefined. Transforming growth factor-β (TGF-β) is a multifunctional cytokine that oversees and directs all aspects of cell development, differentiation and homeostasis, as well as suppresses their uncontrolled proliferation and transformation. Quite dichotomously, tumorigenesis subverts the tumor suppressing function of TGF-β, and in doing so, converts TGF-β to a tumor promoter that stimulates pathophysiological EMT and metastasis. It therefore stands to reason that determining how TGF-β induces EMT in developing neoplasms will enable science and medicine to produce novel pharmacological agents capable of preventing its ability to do so, thereby improving the clinical course of cancer patients. Here we review the cellular, molecular and microenvironmental mechanisms used by TGF-β to mediate its stimulation of EMT in normal and malignant cells.

The epithelium is comprised of highly specialized and diverse cells that play critical roles in nearly all biological processes [1,2]. Indeed, epithelial cells serve as protective barriers that line both the outer (i.e., skin) and inner (i.e., airways, gastrointestinal tract, and so on) body cavities, as well as behave as secretory and glandular tissues. In addition, epithelial cell function varies widely between tissues, and ranges from nutrient absorption in the intestines, to gaseous exchange in the lungs, to lactogenesis in the mammary gland. Equally important is the role of the epithelium in providing the first line of defense against exterior insults and infections, while simultaneously enabling the exchange of vital nutrients needed to maintain tissue homeostasis. The fidelity and function of the epithelium is maintained through its continual renewal and repair, and as such, it is perhaps not surprising to learn that the majority (i.e., ~90% [3]) of cancers arise in cells derived from epithelial origins. Thus, it is imperative that science and medicine uncover the sequence of events that enable specialized and polarized epithelial cells to dedifferentiate along a tumorigenic pathway that terminates in their acquisition of metastatic phenotypes.

Recent evidence has linked the development of tissue fibrosis and cancer metastasis to the inappropriate reactivation of epithelial–mesenchymal transition (EMT), which is the process whereby immotile, polarized epithelial cells transition into highly motile, apolar fibroblastoid-like cells (Figure 1 [1,2,4–6]). Indeed, EMT is a normal physiological process essential for proper embryogenesis and tissue morphogenesis, particularly for the formation of the mesoderm, neural crest, cardiac valve and secondary palate [1,2,7]. With respect to adult tissues, EMT is also engaged in wounded epithelia to facilitate their healing, remodeling and repair in response to tissue damage. Thus, fully differentiated epithelial cells harbor a dormant embryonic transcriptional EMT program that can be reinitiated in response to a variety of specific environmental cues and signals, one of which is the pleiotropic cytokine, transforming growth factor-β (TGF-β). Interestingly, these same cellular and morphological features are observed in cells undergoing pathophysiological EMT, which underlies the development of several human pathologies, such as

Keywords
epithelial–mesenchymal transition ■ metastasis ■ signal transduction ■ transforming growth factor-β ■ tumor microenvironment

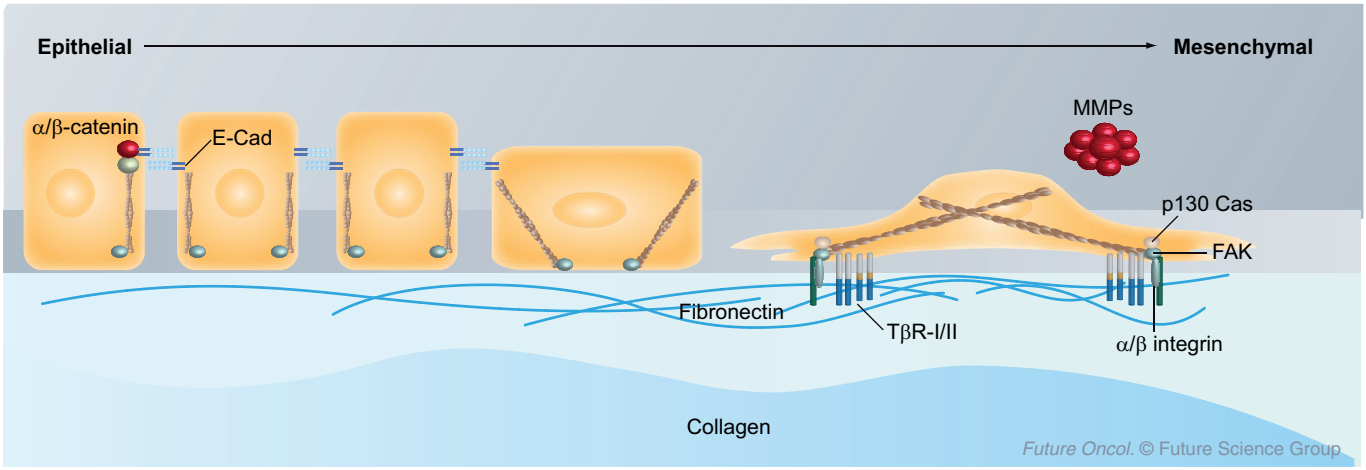

Figure 1. Epithelial cells transition to mesenchymal-like cells in response to TGF-β. This schematic depicts polarized epithelial cells and their cuboidal structure that is maintained via cell–cell junctions comprised of homotypic E-cadherin molecules that are linked to the cortical actin cytoskeleton by α- and β-catenins. TGF-β stimulation of EMT during wound healing or tumor-invasive migration results in the delocalization, degradation and/or downregulation of cell–cell junctions and, consequently, a loss of epithelial integrity. In addition, the morphologic transition of epithelial cells is also supported by the simultaneous formation of actin stress fibers, the upregulation of integrins and the activation of focal adhesion complexes. Moreover, the increased production and secretion of ECM proteins, such as fibronectin and collagen, coupled with the elevated expression and activation of MMPs enables transitioned fibroblastoid-like cells to exhibit invasive and motile phenotypes.
ECM: Extracellular matrix; EMT: Epithelial–mesenchymal transition; FAK: Focal adhesion kinase; MMP: Matrix metalloproteinase; TβR: TGF-β receptor; TGF: Transforming growth factor.

chronic inflammation, rheumatoid arthritis and chronic fibrotic degenerative disorders of the lung, liver and kidney [1,2,4–6,8,9]. Along these lines, aberrant reinitiation of EMT also engenders the acquisition of invasive and metastatic phenotypes in developing and progressing carcinomas, leading to their dissemination and colonization of distant organ sites suitable to support their metastatic growth. A commonality of physiological and pathophysiological EMT is their ability to be induced by TGF-β, which is now recognized as a master regulator of this transdifferentiation process.

TGF-β is a ubiquitously expressed and multifunctional cytokine that not only regulates EMT, but also oversees the development, differentiation and survival of essentially all cell types and tissues [10–13]. TGF-β is also a powerful suppressor of cell growth and proliferation, particularly in cells of epithelial, endothelial and hematopoietic origins [10–13]. Quite dichotomously, aberrations in the TGF-β signaling system regularly take place during tumorigenesis and elicit resistance to its antiproliferative activities, contributing to the formation of human neoplasms. Upon being liberated from the cytostatic activities of TGF-β, cancer cells proliferate, invade and metastasize beyond their tissue of origin when stimulated by TGF-β. How TGF-β suppresses these processes in normal epithelial cells is unclear, as is how TGF-β promotes these processes in their malignant counterparts. Despite the continued uncertainty of the molecular events associated with the diametric activities of TGF-β, it is absolutely clear that this cytokine stimulates the two deadliest aspects of cancer, namely cell invasion and metastasis. Moreover, recent studies indicate that acquisition of metastatic phenotypes by carcinoma cells is critically dependent upon their ability to undergo EMT [4–6,8,14]. Indeed, TGF-β stimulation of EMT was originally demonstrated by Miettinen *et al.* [15], who observed that normal mammary epithelial cells (MECs) acquire fibroblastoid phenotypes in response to TGF-β. In addition, TGF-β3-deficient mice develop cleft palate due to defective palatogenesis associated with aberrant EMT [16]. Similar inactivation of TGF-β2 function impairs endocardial cushion development in chick hearts due to their absence of Slug expression and its ability to activate EMT [17]. Finally, Smad3 deficiency affords protection against EMT-driven retinal [18,19] and renal [20] fibrosis in mice. Thus, these and other seminal studies have clearly established TGF-β as a master regulator of EMT. This review focuses on the myriad of evidence supporting this designation for TGF-β, particularly the cellular, molecular and microenvironmental mechanisms that underlie the ability of TGF-β to induce EMT in normal and malignant cells.

TGF-β signaling & EMT

The general mechanisms whereby TGF-β activates responsive cells and regulates their behavior is depicted in Figure 2 and Figure 3. As shown, transmembrane signaling by TGF-β commences via its binding to three high-affinity receptors, namely the TGF-β type I (TβR-I), type II (TβR-II) and type III (TβR-III or β-glycan). When and where it is expressed, TβR-III clearly is the most abundant TGF-β receptor on the cell surface where it functions as an accessory receptor that binds and presents TGF-β to its signaling receptors, TβR-I and -II, both of which possess intrinsic Ser/Thr protein kinase activity in their cytoplasmic domains [11,12,21–23]. The binding of TGF-β to TβR-II enables the recruitment and activation of TβR-I, leading to its induction of canonical Smad2/3-dependent signaling. Once

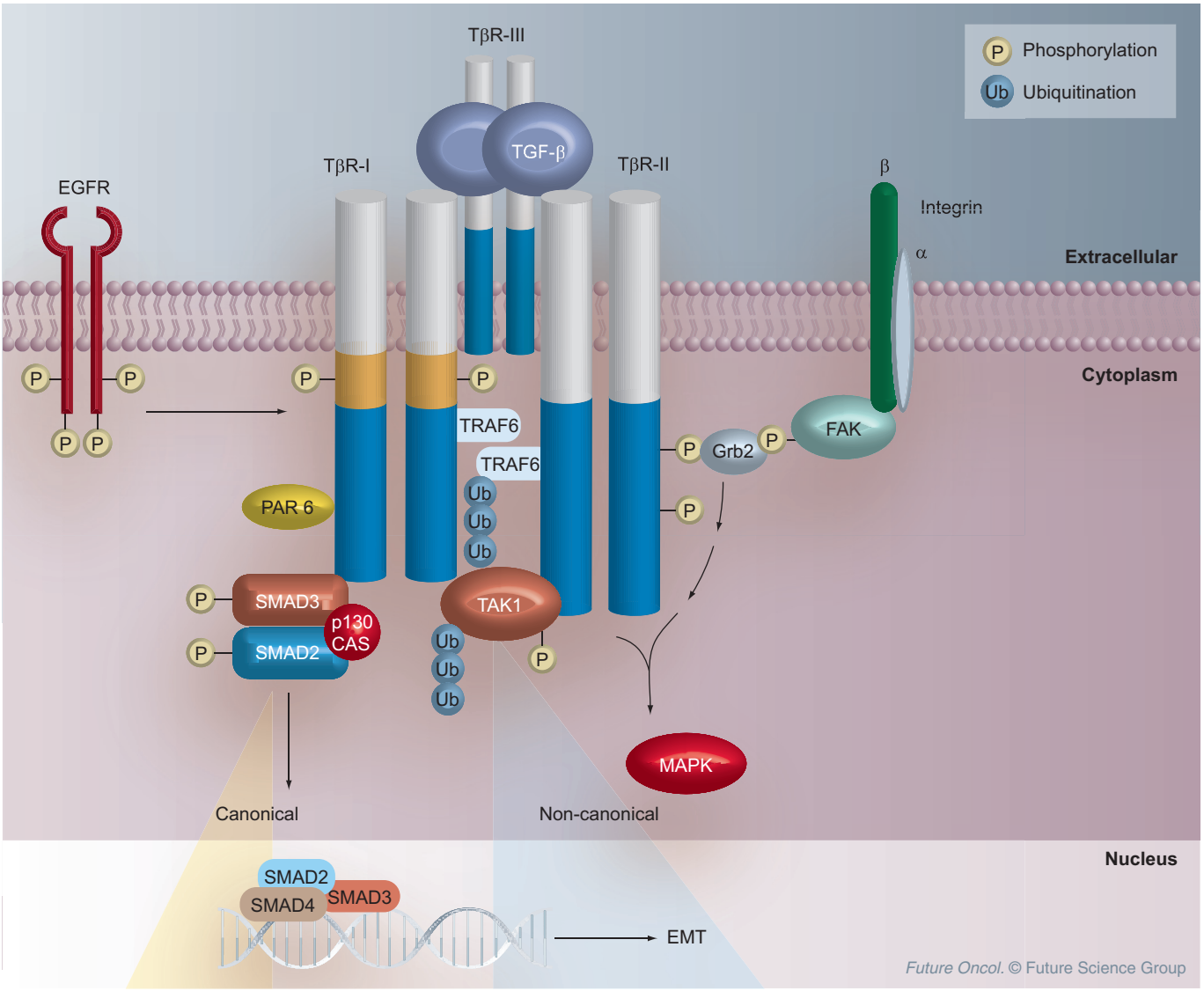

Figure 2. Differential interactions of TGF-β receptors with transmembrane and membrane proximal proteins complexes facilitate the diversity of TGF-β signaling. β1 and β3 integrins interact physically with TβR-II [43–45]. The association of TβR-II with β3 integrin is mediated by FAK, which facilitates the binding of TβR-II to the SH2-binding protein, Grb2. In addition, TβR-II also interacts physically with EGFR [150], which is also activated indirectly by TGF-β through its increased synthesis and secretion of EGFR ligands. The cytoplasmic tails of both TβR-I and TβR-II interact with TRAF6, which ubiquitinates itself and the MAPKKK, TAK1. Additional interactions include the binding of p130Cas to Smad3, as well as that of PAR6 with TβR-I. Importantly, the differential composition of TGF-β receptor and scaffolding complexes directs the coupling of TGF-β to canonical and noncanonical effector activation, as well as underlies the pathophysiological conversion of TGF-β signaling and EMT in malignant epithelial cells. The biological outcomes of these various protein–protein interactions are discussed in the text.
EGFR: Epidermal growth factor receptor; EMT: Epithelial–mesenchymal transition; FAK: Focal adhesion kinase; MAPK: Mitogen-activated protein kinase; TAK: TGF-β-activated kinase; TβR: TGF-β receptor; TGF: Transforming growth factor; TRAF: Tumor necrosis factor receptor-associated factor.

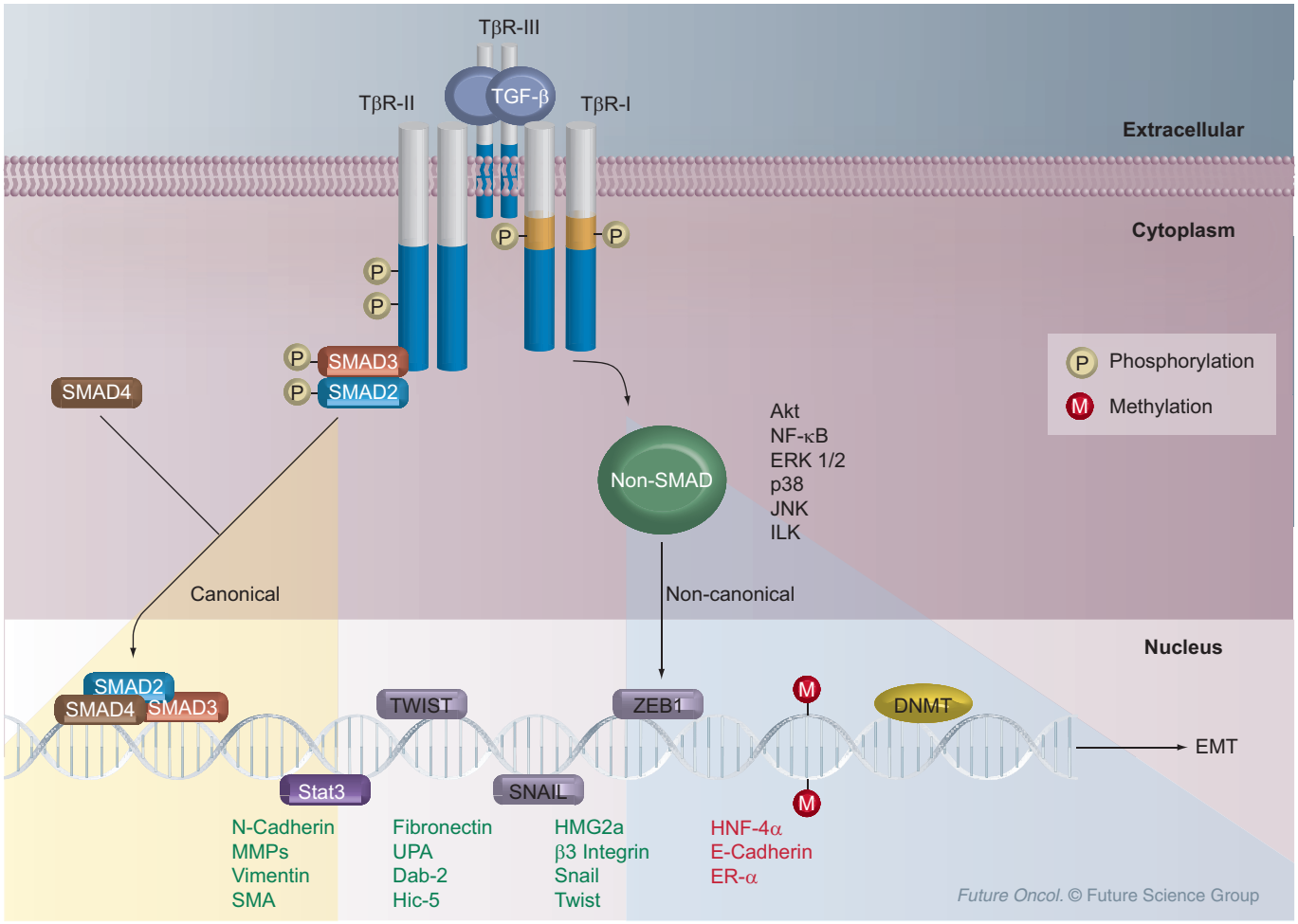

Figure 3. Diverse TGF-β signaling pathways support a complex transcriptional response during EMT. TGF-β stimulates epithelial cells by binding and activating two transmembrane Ser/Thr protein kinase receptors, namely TGF-β type I (TβR-I) and type II (TβR-II). Activation of these ligand:receptor ternary complexes requires TβR-II to transphosphorylate TβR-I, which phosphorylates and activates Smad2/3. Once activated, Smad2/3 form heterocomplexes with Smad4, which collectively translocate to the nucleus to mediate canonical signaling events by TGF-β. Noncanonical TGF-β signaling takes place through its ability to stimulate various alternate signaling pathways discussed in detail herein. Activation of canonical Smad2/3 signaling results in their nuclear translocation with Smad4 and subsequent regulation of gene expression through their numerous interactions with additional transcriptional activators and repressors. Alternatively, activation of noncanonical TGF-β signaling, such as MAP kinases, small GTPases, PI3K/AKT and NF-κB, also couples TGF-β to its regulation of gene-expression profiles operant in mediating EMT. Finally, activation of the transcription factors belonging to the Snail family (e.g., Snail, Twist or ZEB1), or of Stat3 elicit EMT gene expression, which ultimately promotes the prolonged induction of EMT and fibroblastoid-like phenotypes of carcinoma cells via DNA methylation-mediated silencing of E-cadherin expression. Altered coupling of TGF-β to its canonical and noncanonical effector pathways leads to differential gene-expression patterns that ultimately contribute to the development of oncogenic signaling by TGF-β. Indeed, the initiation of oncogenic signaling by TGF-β converts its regulation of physiological EMT in normal epithelial cells to one of pathophysiologic EMT in their malignant counterparts.
EGFR: Epidermal growth factor receptor; EMT: Epithelial–mesenchymal transition; JNK: c-Jun N-terminal kinase; MAP: Mitogen-activated protein; MMP: Matrix metalloproteinase; NF: Nuclear factor; TAK: TGF-β-activated kinase; TβR: TGF-β receptor; TGF: Transforming growth factor.

activated, Smad2/3 form heterocomplexes with Smad4 and translocate into the nucleus, where they regulate the cell-type-specific expression of TGF-β-responsive genes [11,12,21–23]. It is interesting to note that the variety of cell responses exhibited in response to TGF-β are governed primarily by the cell-type-specific expression of various Smad2/3-interacting transcription factors (e.g., AP-1 and Forkhead family members, Stats, and so on [11,22]), as well as their association with additional transcriptional activators or repressors [11,12,21–23]. Moreover, the amplitude and duration of Smad2/3 signaling is modulated by several mechanisms, including the expression of adapter and/or anchoring proteins SARA [24], Hgs [25] and Dab2 [26], that enable Smad2/3 phosphorylation by TβR-I, and the inhibitory Smad, Smad7, which prevents the phosphorylation of Smad2/3 [27–29] and induces the degradation of TGF-β receptors [30,31]. In addition, the

inhibitory functions of Smad7 are regulated by its interaction with STRAP [32], which potentiates the anti-TGF-β activity of Smad7, and by its association either with AMSH2 [33] or Arkadia [34–36], both of which negate the anti-TGF-β activity of Smad7. As alluded to above, the activation of Smad2/3 by TGF-β represents the canonical TGF-β signaling system, which is shown diagrammatically in Figure 3.

Also depicted in Figure 3 is the coupling of TGF-β to a variety of noncanonical signaling systems, including:

- The mitogen-activated protein (MAP) kinases ERK1/ERK2, p38 MAPK and c-Jun N-terminal kinase (JNK);
- The growth and survival kinases PI3K, AKT/PKB and mTOR;
- The small GTP-binding proteins Ras, RhoA, Rac1 and Cdc42 [37–45].

In addition, TGF-β typically represses nuclear factor (NF)-κB activity in normal epithelial cells [46,47], but readily activates this transcription factor in their malignant counterparts [47–51]. More recently, TGF-β has been shown to activate a number of protein tyrosine kinases (PTKs), including focal adhesion kinase (FAK) [52,53], Src [43–45,54], and Abl [55,56], which results in the inappropriate amplification of noncanonical TGF-β signaling in mesenchymal or dedifferentiated epithelial cells. Moreover, imbalances in the activation status of canonical and noncanonical TGF-β signaling systems may very well underlie the ability of TGF-β to induce EMT in normal and malignant cells. The importance of canonical and noncanonical TGF-β signaling systems to promote physiological and pathophysiological EMT is presented in greater detail below.

Defining EMT

The phenomenon of EMT is defined by the morphologic and genetic transition of epithelial cells to fibroblastoid- or mesenchymal-like cells. An inherent characteristic or hallmark of EMT, including that stimulated by TGF-β, is the dramatic phenotypic change in epithelial cell morphology [4–6,8,14]. Typically, fully differentiated epithelium manifests as a single layer of polarized epithelial cells comprised of well-defined apical and basolateral surfaces, as well as a clearly demarcated actin cytoskeleton arranged into discrete 'cobblestones' that reflect regions of concentrated actin fibers at cell–cell junctions. In response to the initiation of EMT, cell–cell junctions disassemble and filamentous actin undergoes a dramatic redistribution to form prominent stress fibers, which is tracked experimentally via the use of a fluorescently labeled mushroom toxin, phalloidin. The combined effect of these various cell biological activities is a loss of epithelial cell polarity (Figure 1).

Examining the biochemical and molecular alterations in cell–cell junction formation and dissolution has enabled science and medicine to garner a more complete assessment of the events underlying EMT. Indeed, a number of recent examinations have elucidated a variety of molecular complexes and scaffolds that govern the development of cell–cell junctions, including tight junctions, adherens junctions and desmosomes [5]. Not surprisingly, a series of coordinated and dynamic processes underlie formation of these macromolecular complexes during the development and maintenance of the epithelium, while changes in the expression and localization of junctional proteins constitute useful measures to track the progression of EMT. For instance, tight junctions are formed by the actions of the transmembrane proteins, claudins, occludins and junctional adhesion molecules (JAMs), which are linked to the actin cytoskeleton via the scaffold proteins ZO-1, -2 and -3 [57,58]. Moreover, following their formation, tight junctions and their constituents play essential roles in regulating the biology, homeostasis and architecture of epithelial cells, and in preventing the initiation of EMT and tumorigenesis [59]. By contrast, the initiation of EMT induces a drastic modulation of tight-junction localization in epithelial cells [15,38]. For instance, the function of partitioning-defective 6 (Par6), which governs the formation of tight junctions, the establishment of apical–basolateral polarity, and the initiation of polarized cell migration [60], is compromised by its physical interaction with TβR-I and subsequent phosphorylation by TβR-II in epithelial cells stimulated with TGF-β [61]. Once phosphorylated, Par6 recruits and interacts with the E3 ubiquitin ligase, Smurf1, which ubiquitinates the small GTPase, RhoA, leading to its degradation and subsequent dissolution of tight junctions during EMT stimulated by TGF-β [62]. The importance of Par6 to EMT induced by TGF-β is highlighted by the ability of TβR-II-resistant Par6 mutants (i.e., S345A-Par6) to prevent MECs from undergoing EMT in response to TGF-β [61].

Unlike tight junctions, adherens junctions consist of transmembrane E-cadherin (Epithelial-cadherin) proteins that are linked

to the actin cytoskeleton by α- and β-catenins [63]. TGF-β stimulation of EMT represses E-cadherin transcription (discussed below), as well as disrupts its localization at the plasma membrane in part via diminished activation of the small GTPase, Rac1 [62]. The net effect of altered E-cadherin function during EMT is the dissolution of adherens junctions. In addition, the loss of cell–cell contacts parallels the development of prominent actin filaments and the appearance of fibroblastoid-like phenotypes in transitioning epithelial cells, processes requiring the activation of RhoA by TGF-β [64,65]. The mechanisms underlying TGF-β regulation of adherens junction expression and function are discussed below.

EMT, TGF-β & cell microenvironments

Maintaining homeostasis within cell microenvironments is essential to alleviating disease development in humans, particularly cancer. Tumor development has been likened to that of dysfunctional miniature organs that house a mixture of malignant and normal cells, including fibroblasts, endothelial and immune cells [66]. It is also important to remember that the growth and progression of tumors are not inherent properties of the cancer cells themselves, but instead are dictated in large part by a delicate balance between positive and negative proliferative signals produced by diverse cell types within tumor microenvironments. Indeed, alterations within tumor microenvironments can either suppress or promote cancer progression in a manner that mirrors the acquisition of oncogenic signaling by TGF-β in developing neoplasms. Biologically, TGF-β is a master inhibitor of cell-cycle progression; however, this cytokine also functions as a master regulator of extracellular matrix (ECM) production, deposition and remodeling, all of which are essential processes during EMT. Along these lines, recent evidence has shown that TGF-β stimulation of cancer progression proceeds in part via its reprogramming of cell microenvironments, particularly by its ability to target the behaviors of neighboring endothelial cells (ECs) and fibroblasts. Moreover, ECs and fibroblasts typically respond to TGF-β by synthesizing and secreting numerous cytokines, growth factors and ECM components capable of driving the progression of tumors from indolent to aggressive states [67,68]. A vital component of normal and malignant cell microenvironments is the ECM, which functions as a gel-like structural scaffold for cells comprised of polysaccharides and fibrous proteins, including collagen, fibronectin and elastin; as well as being a molecular sensor that monitors, detects and responds rapidly to physiological and pathophysiological changes within cell microenvironments. Indeed, under physiological conditions, the ECM serves as a storage reservoir that sequesters numerous growth factors and cytokines that can be rapidly released in response to ECM perturbations or insults, thereby circumventing the need for *de novo* protein synthesis to elicit biological behaviors [69]. Thus, the microenvironment of epithelial cells plays a critical role in maintaining their polarization and differentiation, processes that are disrupted temporarily during physiological EMT and its modification of epithelial cell microenvironments. By contrast, chronic disruptions within carcinoma cell microenvironments elicits pathologic EMT and its ability to support cancer cell invasion and metastasis. Table 1 identifies numerous EMT-associated genes whose expression is regulated by TGF-β, and readers desiring more in-depth discussions of the activities and functions of these genes in governing EMT and epithelial cell biology are directed to several recent reviews [1,2,4–6]. In the following sections, we highlight many of the mechanisms that underlie the ability of TGF-β to induce EMT and its associated alterations within the microenvironments of transdifferentiating cells.

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) comprise a large family of proteases that regulate essential steps of embryogenesis and tissue morphogenesis, and of wound healing and cell growth. MMPs also possess the ability to degrade nearly all ECM and basement membrane components, as well as the ability to promote the development and progression of human malignancies [70,71]. Along these lines, TGF-β enhances the tumorigenicity and invasiveness of breast cancer cells by inducing their expression of MMP-2 and -9 [72,73], which is consistent with the general importance of upregulated MMP expression in mediating the acquisition of invasive phenotypes in several cancers [74]. Indeed, aberrant MMP expression (e.g., MMP-7 or matrilysin) facilitates the development of mammary fibrosis and desmoplasia, which increase tumor rigidity and the selection, expansion and dissemination of metastatic cells [75,76]. Similarly, upregulated MMP-3 expression is sufficient to induce lung and mammary fibrosis [77,78], and to stimulate EMT in carcinomas [79]. Thus, elucidating the connections between aberrant

Table 1. Expression of EMT-associated genes targeted by TGF-β.

Study	Gene name	Expression change	Ref.
Miettinen *et al.* (1994)	E-cadherin	Decrease	[15]
Galliher *et al.* (2006)	β3 integrin	Increase	[43]
Hazan *et al.* (2000)	N-cadherin	Increase	[112]
Lehembre *et al.* (2008)	*NCAM*	Increase	[85]
Duivenvoorden *et al.* (1999)	*MMP-2*	Increase	[72]
Radisky *et al.* (2005); Farina *et al.* (1998)	*MMP-3*	Increase	[79,236]
Kim *et al.* (2007); Farina *et al.* (1998)	*MMP-9*	Increase	[73,236]
Grunert *et al.* (2003)	Vimentin	Increase	[117]
Masszi *et al.* (2003)	α-Smooth muscle actin	Increase	[118]
Ignotz *et al.* (1986)	Fibronectin	Increase	[102]
Dhasarathy *et al.* (2007)	Estrogen receptor-α	Decrease	[203]
Farina *et al.* (1998)	Urokinase plasminogen activator	Increase	[236]
Hocevar *et al.* (2001)	*Dab2*	Increase	[26]
Tumbarello *et al.* (2007)	*Hic5*	Increase	[157]
Thuault *et al.* (2006)	*HMG2A*	Increase	[111]

EMT: Epithelial–mesenchymal transition.

MMP expression and the development of fibrosis and/or EMT will offer important clues as to how EMT promotes cancer progression. For instance, does pathophysiologic EMT solely mediate the acquisition of invasive phenotypes by developing carcinomas, or does this event simply reflect the transdifferentiation of a subset of carcinoma cells into tumor supporting stroma cells (e.g., myofibroblasts) [80]? Indeed, tumor-associated myofibroblasts upregulate their production and secretion of TGF-β, which may serve in establishing a positive-feedback loop that drives the selection and expansion of metastatic carcinoma cells [81–83]. Collectively, these findings point to the need for additional studies to fully address these questions, particularly since the expression and activity of MMPs alters the expression of E-cadherin, Snail, vimentin and TGF-β in a manner consistent with the induction of EMT [79].

Neuronal cell adhesion molecule

Neuronal cell adhesion molecule (NCAM) is a member of the immunoglobulin superfamily and has been implicated as a mediator of tumor progression and metastasis [84]. Recently, TGF-β stimulation of EMT was observed to induce NCAM expression in a manner correlated with downregulated expression of E-cadherin [85]. Functionally, upregulated expression of NCAM during EMT facilitates the formation of β1 integrin-containing focal adhesion complexes [85]. Interestingly, the extracellular domain of NCAM is cleaved proteolytically by MMP-28 (epilysin), which also induces EMT through its ability to activate latent TGF-β complexes from inactive ECM depots [86]. In addition, MMP-28 expression is also upregulated in a EMT-dependent manner in wounded epithelial cells, and in metastatic breast cancer cells [87]. Thus, future studies need to determine the physiological and pathophysiological connections between NCAM, MMP-28 and TGF-β during the initiation of EMT in normal and malignant epithelial cells.

Urokinase plasminogen activator

Urokinase plasminogen activator (uPA) is a serine protease whose elevated expression in human cancer correlates with advanced disease states and poor clinical outcomes, presumably through its ability to promote cancer cell invasion and metastasis [88,89]. Accordingly, uPA expression is essential for breast and ovarian cancer metastasis in mice [90,91], and for hypoxia-induced EMT in breast cancer cells via uPA receptor-mediated activation of AKT and Rac1 [92]. TGF-β is a potent inducer of uPA expression, yet the role of this event in mediating EMT and metastasis stimulated by TGF-β remains to be elucidated fully. Recently, the activation of JNK1/2 was shown to be essential for TGF-β stimulation of uPA expression and EMT [93], which is consistent with the notion that noncanonical TGF-β signaling promotes its oncogenic activities in epithelial cells.

Plasminogen activator inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1) is an antagonist of tissue-type plasminogen activator (tPA) and uPA, as well as a physical interactor of the ECM ligand, vitronectin [94,95]. tPA and uPA both activate the serine protease activity of plasminogens (or plasmins), resulting in the degradation of blood plasma proteins, such as fibrin and von Willebrand factor, and of ECM proteins, such as fibronectin, thrombospondin and laminin [95]. Through its ability to inhibit tPA and uPA, PAI-1 prevents the activation of intravascular and cell-associated plasminogen, and as such, impedes the breakdown of blood clots and ECM proteins necessary to enable carcinoma cells to undergo invasion and extravasation reactions during metastasis [95].

TGF-β is principal player involved in stimulating PAI-1 transcription in part via activation of p53, which binds and stabilizes PAI-1 transcripts [96,97]. Quite dichotomously, overexpression of PAI-1 has been observed to reduce the migration and invasion of breast and ovarian cancers [94,98]; however, PAI-1 polymorphisms or its aberrantly elevated expression have also been associated with a poor prognosis and the increased risk of metastasis in breast cancer patients [99]. Thus, the precise mechanisms underlying the dynamic relationship between PAI-1, plasminogen and TGF-β regulatory loops, as well as their impact on cancer cell motility, remain an active and important topic of investigation.

Collagen

Collagen is an abundant ECM molecule that assembles into tensilely strong fibers that provide mechanical support to tissues. The major types of collagen, types I–IV, are distributed differentially in specific tissues of the body. For instance, collagen IV is a major component of the basal lamina, a specialized component of the basement membrane in the mammary gland. Invading breast cancer cells must degrade collagen IV to migrate into surrounding tissue. Interestingly, Endo180 is a cell-surface receptor that promotes the uptake of collagen for its degradation intracellularly. Moreover, Endo180 expression is elevated significantly in highly invasive breast cancer cells; induced transcriptionally by TGF-β stimulation in breast cancer cells; and reduces the collagen content and enhances the growth of mammary tumors produced in mice [100]. In addition, TGF-β also governs collagen function by upregulating the expression of MMP-2 and other collagenases in normal and malignant MECs, leading to their enhanced migration and invasion [72,73,101].

Fibronectin

Fibronectin is a large and critical ECM glycoprotein whose elevated production by cancer cells is classically associated with the acquisition of EMT, and more recently, with the development of the metastatic niche [67]. TGF-β is a potent inducer of fibronectin production and deposition into the ECM [102], where it binds integrins and regulates cell adhesion and motility. The synthesis and secretion of fibronectin into the ECM is primarily mediated by fibroblasts, and by epithelial cells induced to undergo EMT (Table 1) [103]. With respect to the latter, nontumorigenic EpH4 MECs engineered to express oncogenic Ras (i.e., EpRas cells) significantly upregulate their expression of fibronectin and its receptor, α5β1 integrin, when stimulated with TGF-β [104]. More importantly, administration of neutralizing α5 integrin antibodies to TGF-β-treated EpRas cells inhibited their migration and induced a significant apoptotic response [104]. Thus, the synthesis and deposition of fibronectin, coupled with changes in expression and activation of integrins (see below), clearly represent an important mechanism that enables TGF-β to stimulate invasive migration during EMT.

Cadherin switching

A phenotypic hallmark of EMT is its ability to downregulate the expression and function of E-cadherin, which is critical in mediating epithelial cell integrity and cell–cell adhesion [105]. Reduced E-cadherin expression in developing and progressing carcinomas takes places through several mechanisms that function en masse to promote cancer cell invasion [5]. For instance, E-cadherin can be inactivated by genetic mutations, and humans harboring these E-cadherin mutations have significantly increased risk of developing cancer [106]. In addition, epigenetic silencing of the E-cadherin *(CDH1)* promoter via hypermethylation of its 5′ CpG island also enhances the development of carcinomas [107]. Along these lines, TGF-β stimulation of EMT also represses the synthesis of E-cadherin transcripts in large part via its ability to induce the expression of the Snail/ZEB family of basic helix-loop-helix transcription factors, including that of Snail1, ZEB1, Snail2/Slug, Twist and ZEB2/SIP1 [105,108–110]. Although the relative contribution of canonical and noncanonical TGF-β signaling in mediating transcriptional activation of these E-cadherin repressors remains to be determined definitely, recent evidence suggests that these

events do take place in a cell type-specific manner in response to TGF-β. For example, activation of Smad2/3 by TGF-β in MECs induces their expression of the nuclear high mobility group A2 (HMGA2), which promotes EMT by stimulating the expression of Snail1, Snail2/Slug and Twist, and by inhibiting the expression of inhibitor of differentiation 2 (ID2) [111]. In addition, while the functional consequences of diminished E-cadherin expression on the behavior of transitioning epithelial cells is well established, recent studies have determined that these same cells also exhibit upregulated expression of N-cadherin (i.e., neuronal-cadherin) [65], an event linked to elevated cell motility and poor clinical outcomes in cancer patients [112–114]. At present, the necessity of increased N-cadherin expression in mediating EMT, particularly that stimulated by TGF-β, remains to be clarified. Indeed, we [45] and others [115,116] recently established murine 4T1 breast cancer cells as a model of advanced-stage breast cancer whose increased malignancy is governed by TGF-β. Interestingly, while 4T1 cells undergo EMT and downregulate E-cadherin when stimulated by TGF-β [44,45], these cells fail to express and/or elevate their expression of N-cadherin during EMT initiated by TGF-β [Wendt MK, Schiemann WP. University of Colorado Denver, CO, USA. Unpublished data]. Thus, future studies aimed at determining the exact nature of N-cadherin in promoting the acquisition of EMT and metastatic phenotypes are clearly warranted.

Vimentin

The intermediate filament protein vimentin is expressed in all primitive cell types, but not in their differentiated counterparts. In light of its role as a master regulator of EMT, it perhaps is not surprising to learn that TGF-β stimulation of EMT reactivates vimentin expression in dedifferentiating epithelial cells, an event that serves as a canonical marker for detecting fully transitioned epithelial cells and their acquisition of fibroblastoid-like phenotype [117].

α-smooth muscle actin

A major component of contractile microfilaments is α-smooth muscle actin (α-SMA), which also serves as a canonical marker for detecting fibroblasts/mesenchymal cells, particularly myofibroblasts. Indeed, during its induction of EMT, TGF-β stimulates α-SMA expression in transitioning epithelial cells [118], an event associated with increased tumor invasion and decreased patient survival rates [119].

Transmemberane & membrane proximal protein complexes that impact TGF-β signaling & EMT

Recent evidence suggests that cell-surface signaling receptors, such as receptor tyrosine kinases (RTKs) and G-protein-coupled receptors, do not function in isolation and instead require accessory signaling inputs that arise from interacting receptors and signaling modules. As shown in Figure 2, the function and behavior of TGF-β receptors are also modulated via their association with an ever-expanding array of receptor-interacting molecules and scaffolding proteins. Included in this growing list of TGF-β receptor regulators are members of the integrin superfamily of heterodimeric transmembrane adhesion receptors, which function as direct physical conduits that link the ECM to the cytoskeleton of the cell [120,121]. Integrin signaling commences upon their clustering and subsequent stimulation of the Ser/Thr protein kinase integrin-linked kinase (ILK), as well as members of the Src family of PTKs and FAK, leading to the activation of a vast array of downstream effectors, including members of the MAP kinase family of protein kinases, members of the Ras/Rho family of small GTPases and members of the PI3K and AKT signaling axes [120–124]. Integrins also regulate cell behavior through their ability to form complexes with RTKs [125,126]. For instance, β1 integrins form FAK-dependent complexes with the receptors for EGF, PDGF and HGF [126,127], and in doing so, enable growth factor-mediated induction of cell migration and invasion [126]. Interestingly, the scaffolding function of FAK is independent of its PTK activity, but does require its N-terminal FAK Ezrin Radixin Moesin (FERM) domain and C-terminal focal adhesion targeting (FAT) domain to bind RTKs and β1 integrins, respectively [126]. Lastly, the establishment of EMT phenotypes in cultured cells, as well as the development of late-stage cancers and their acquisition of invasive and metastatic phenotypes, both have been linked to dramatic changes in the expression and localization of integrins in epithelial cells [104,128].

In addition to its regulation of cell-cycle progression, TGF-β also figures prominently in mediating ECM remodeling and repair via its ability to regulate integrin expression [129–131]. Moreover, αvβ6 and αvβ8 integrin ligation promotes the activation of TGF-β1 and TGF-β3 from inactive ECM depots [132–137], which regulates alveolar development, wound closure, fibrosis and EMT [130,132,138–140]. In addition, epidermal transgenic expression of α6β4 integrin also elicits elevated

development of metastatic papillomas and carcinomas in a chemical carcinogenesis model of skin cancer. Importantly, the tumorigenicity associated with α6β4 integrin expression was linked to its ability to uncouple TGF-β from activating Smad2/3 and preventing cell-cycle progression [141]. Similar reciprocity between integrins and TGF-β is observed in cancers of the prostate, whose metastasis to bone is stimulated by TGF-β and its induction of α2β1 integrin, which binds to bone-derived type I collagen [142]. Thus, the ability of TGF-β to stimulate cancer progression and metastasis requires an intricate interplay between signals arising from TGF-β receptors and those initiated by integrins. Accordingly, integrins have been found to associate with TGF-β receptors and play a critical function in coupling TGF-β to activation of its noncanonical effectors, and to its induction of EMT. For instance, neutralizing β1 integrin antibodies abrogated the ability of TGF-β to activate p38 MAPK and induce EMT in MECs [39]. Similarly, hepatocellular carcinoma cells elevate their expression of α3β1 integrin in response to TGF-β, an event that enhances their motility and invasiveness [143]. Moreover, administering laminin-5 together with TGF-β stimulated hepatocellular carcinoma cells to undergo EMT in an α3 integrin-dependent manner [144], further demonstrating the necessity of integrins to cooperate with TGF-β to induce EMT and invasion in transitioning cells.

We also described the functional cooperation between integrins and TGF-β in promoting EMT, as well as in stimulating the development and progression of mammary tumors. For instance, we found the expression and activity of αvβ3 integrin and its downstream effector Src to be essential for TGF-β stimulation of MEC proliferation, invasion and EMT [43–45]. In addition, transgenic expression of αvβ3 integrin not only negated the cytostatic response of normal MECs to TGF-β, but also enhanced its stimulation of MEC invasion and p38 MAPK activation. Importantly, inactivation of either αvβ3 integrin or Src function abolished the ability of TGF-β to stimulate EMT and invasion in normal and malignant MECs [43,44]. Mechanistically, β3 integrin interacts physically with TβR-II, leading to its:

- Phosphorylation on Y284 by Src;
- Interaction with Grb2 and Shc at phosphorylated Y284;
- Activation of p38 MAPK;
- Stimulation of EMT and invasive migration in normal and malignant MECs [44].

Along these lines, the growth and metastasis of breast cancer cells in mice absolutely required TβR-II to be phosphorylated on Y284, a phosphotransferase reaction that disrupts the delicate balance between canonical and noncanonical TGF-β signaling inputs activated during mammary tumorigenesis [45]. In addition to its ability to promote pulmonary metastasis stimulated by TGF-β [45], αvβ3 integrin expression also directs breast cancer cell metastasis to bone [145,146] and lung [146], in part through a TGF-β-dependent pathway. Collectively, these findings suggest that pharmacological targeting of noncanonical TGF-β effectors, particularly αvβ3 integrin, Src and p38 MAPK, may prove efficacious in treating metastatic breast cancers.

Besides integrins, a growing number of intracellular proteins have also been shown to interact with and regulate the activity of TGF-β receptors. For instance, two members of the focal adhesion complex, namely FAK and its downstream effector p130Cas (p130Crk-associated substrate), both influence the cellular response to TGF-β through dramatically different mechanisms. Indeed, TGF-β stimulates FAK and its relative PYK2 during EMT [147], leading to the activation of JNK and the subsequent upregulation α-SMA in fibroblasts [148]. In addition, FAK activation in hepatocytes is necessary for the downregulation and declocalization of E-cadherin from the plasma membrane [149]. Finally, we recently established FAK as a molecular scaffold that facilitates the formation of oncogenic β3 integrin: TβR-II complexes and their activation of Src and interaction with Grb2 [150]. Moreover, the ability of FAK to form these signaling complexes is essential for TGF-β stimulation of p38 MAPK in breast cancer cells, as well as for their induction of EMT and metastasis stimulated by TGF-β [45,150]. Thus, the aberrant recruitment of FAK to TGF-β receptors readily influences the oncogenic conversion of TGF-β from a tumor suppressor to a tumor promoter, including its stimulation of pathophysiological EMT in carcinoma cells.

In stark contrast to FAK, the incorporation of p130Cas into active TGF-β receptor complexes alters the coupling of TGF-β to the canonical Smad2/3 pathway. Indeed, the activation and phosphorylation of p130Cas following cellular adhesion to ECM matrices led to its association and inactivation of Smad3, and to diminished cytostatic activity of TGF-β [151]. Similarly, we find that rendering malignant, metastatic MECs deficient in p130Cas enhances Smad2/3 activation by TGF-β, but fails to alter its coupling to

p38 MAPK; however, this same cellular condition selectively inhibited breast cancer metastasis only in cells that possessed heightened TGF-β signaling [Wendt MK, Schiemann WP. University of Colorado Denver, CO, USA. Unpublished data], suggesting that p130Cas acts as a molecular integrator of canonical Smad2/3 signaling when confronted with elevated oncogenic behavior mediated by the receptors for TGF-β or EGF [152].

Recently, the regulation of TGF-β signaling has been shown to be modulated by two additional adapter proteins that localize to focal adhesions, namely Hic5 and Disabled-2 (Dab2). Indeed, Hic5 is a member of the paxillin superfamily and, like paxillin, functions as an adapter protein at focal adhesions [153], as well as resides in the nucleus where it functions as a transcriptional coactivator in regulating gene expression induced by the androgen [154] and glucocorticoid receptors [155,156]. Moreover, Hic5 expression is low in quiescent MECs, but is induced rapidly via a RhoA/ROCK-dependent pathway following administration of TGF-β [153]. In addition, uncoupling Hic5 from TGF-β regulation prevents its induction of EMT in normal MECs [157]. Thus, Hic5 plays an essential role in coupling TGF-β receptors to activation of RhoA/ROCK and, consequently, to the induction of EMT. Along these lines, Dab2 was identified originally as an ovarian tumor suppressor gene [158,159] that regulates the actin cytoskeletal architecture during cell migration and adhesion [160]. More recently, Prunier *et al.* [161] established Dab2 as a novel gene target of TGF-β in MECs undergoing EMT in part via its ability to:

- Associate with TGF-β receptor complexes [26]
- Promote Smad2/3 activation by TGF-β receptors [26]
- Stimulate the activation of TAK1 and JNK, which induced fibronectin expression and enhanced cell motility [162]

Along these lines, TGF-β stimulates Dab2 expression in MECs undergoing EMT, which promotes the formation of Dab2:β1 integrin complexes and their activation of FAK [161]. Importantly, measures capable of disrupting Dab2 function prevents EMT stimulated by TGF-β, as well as promotes its ability to induce apoptosis in MECs. Although the molecular mechanisms underlying the ability of TGF-β to stimulate Dab2 expression remains to be defined, these studies do provide interesting insights into the connections that govern alterations in cell survival and morphology regulated by TGF-β.

Finally, two laboratories recently identified a novel collaboration between signaling molecules activated by TNF-α and those activated by TGF-β. Indeed, both studies demonstrated the ability of TGF-β to induce the physical association of its receptors with that of TNF receptor-associated factor 6 (TRAF6) [163,164], leading to K63-linked polyubiquitination and activation of TAK1 and its subsequent stimulation of p38 MAPK and JNK. Moreover, whereas TRAF6 deficiency had no effect on the coupling of TGF-β to Smad2/3, this same cellular condition uncoupled TGF-β from activation of MAP kinases and prevented this cytokine from inducing EMT in normal MECs [164]. Taken together, these studies reinforce the notion that imbalances in the TGF-β signaling system that favor its activation of noncanonical effectors over that of its canonical Smads are crucial to its induction of EMT in normal and malignant epithelial cells. These findings also point to the need for additional studies to define precisely how these aberrant protein complexes and modules impact the epithelial cell response to TGF-β, and how science and medicine can better target these effector molecules that promote oncogenic signaling and EMT initiation by TGF-β.

Signaling systems involved in EMT stimulated by TGF-β

Transmembrane signaling by TGF-β is traditionally associated with its activation of Smad2/3 and their ability to alter the transcription of TGF-β-responsive genes, which clearly play an important role in mediating the ability of TGF-β to induce EMT, tumor formation and cancer cell metastasis [165]. The necessity of Smads 2 and 3 for TGF-β stimulation of EMT has been reviewed extensively in the scientific literature, and readers desiring a more in-depth description of Smad2/3 function in regulating EMT in normal and malignant cells are directed to several recent reviews [4,5,11]. As alluded to above, the enhanced coupling of TGF-β to its noncanonical effectors figures prominently in mediating its biological and pathological behaviors, particularly its ability to induce EMT and cancer cell metastasis. Table 2 lists a variety of noncanonical effectors targeted by TGF-β during its activation of EMT, while the role of these signaling molecules during epithelial cell EMT induced by TGF-β is discussed below.

Rho family of small GTPases

The Rho family of small GTPases is comprised of RhoA, Rac1 and Cdc42, which regulate the formation of stress fibers, lamellipodia or filopodia,

Table 2. Signaling pathways activated during epithelial–mesenchymal transition stimulated by TGF-β.

Study	Pathway	Ref.
Piek *et al.* (2001)	Smad2/3	[237]
Bhowmick *et al.* (2001)	Rho family of small GTPases	[65]
Bakin *et al.* (2000)	PI3K and AKT	[38]
Huber *et al.* (2004)	NF-κB	[180]
Xie *et al.* (2004)	ERK1/2	[184]
Bhowmick *et al.* (2001); Galliher & Schiemann (2006; 2007; 2008)	p38 MAPK	[39,43–45]
Hocevar *et al.* (1999)	JNK	[186]
Lin *et al.* (2007); Lee *et al.* (2004)	Integrin-linked kinase	[91,178]

ERK: Extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase; NF: Nuclear factor; TGF: Transforming growth factor.

respectively [166,167]. Indeed, Rac1 is an established inducer of cell–cell adhesions in epithelial cells [168], which contrasts sharply with the ability of RhoA to dissolve these adhesive complexes to facilitate times of cell migration [62]. Given the importance of these small GTPases in overseeing cell adhesion, morphology and migration, it is fitting to find that these effectors are intimately involved in EMT stimulated by TGF-β. For instance, the activation of RhoA by TGF-β enables MECs to undergo EMT, while measures capable of inhibiting RhoA function or that of its downstream effector, $p160^{ROCK}$, uncouple TGF-β from EMT in MECs [65]. Moreover, RhoA activation is also essential for TGF-β stimulation of α-SMA expression in renal epithelial cells undergoing EMT [118]; however, completion of this same cellular event in lens epithelial cells requires signaling inputs from both RhoA/ROCK and Smad2/3 [169]. Taken together, these studies point to the overall importance of noncanonical TGF-β signaling, particularly that induced by RhoA/ROCK, to induce EMT in epithelial cells.

PI3K/AKT

The tumor-suppressing activity of TGF-β not only reflects its ability to induce cytostasis, but also its propensity to activate apoptosis in epithelial cells [10,11,13,170]. Interestingly, the ability of TGF-β to stimulate apoptosis is frequently subverted during tumorigenesis, leading to enhanced cancer cell survival via activation of the PI3K and AKT signaling systems by TGF-β. Indeed, administration of PI3K inhibitors to MECs inhibits their activation of AKT and ability to undergo EMT in response to TGF-β [38]. The activation of AKT by TGF-β can transpire directly via TGF-β receptors or indirectly via the transactivation of EGF [171] and PDGF [172] receptors, which induce the expression of genes operant in mediating cancer cell EMT, metastasis and survival. In addition to altering gene-expression profiles, AKT also regulates mRNA translation when impacting the response of epithelial cells to TGF-β. For instance, TGF-β stimulation of EMT in MECs is accompanied by an increase in cell size and protein content, both of which correlate with the rapid activation of mammalian target of rapamycin (mTOR) in transitioning MECs [40]. Somewhat unexpectedly, administering the mTOR inhibitor, rapamycin, to MECs failed to affect their acquisition of an EMT morphology in response to TGF-β; however, this same cellular condition completely prevented the ability of TGF-β to increase MEC size and protein production, as well as inhibited their migration and invasion [40]. Taken together, these findings highlight an important bifurcation in the TGF-β signaling system that dissociates the ability of TGF-β to alter cell morphology from its ability to elevate cell motility. Future studies need to identify the transcriptional and translational objectives targeted by TGF-β, as well as determine their relative contribution to oncogenic signaling stimulated by TGF-β in normal and malignant cells.

Integrin-linked kinase

In addition to their stimulation of PTKs, the ECM engagement of β1 and β3 integrins also activates the Ser/Thr protein kinase integrin-linked kinase (ILK) and its ability to mediate the stimulation of MAP kinases, PI3K/AKT and small GTPases; and the inhibition of GSK3β [173–175]. Accordingly, targeting ILK expression to mouse mammary glands elicited a hyperplastic reaction that progressed to full-blown breast cancer in part via constitutive activation of ERK1/2 and AKT, which inactivated GSK3β [176]. Elevated ILK expression is associated with the acquisition of EMT phenotypes by MECs, including reductions in their expression of E-cadherin and adhesion, as well as increases in their formation of actin stress fibers and invasion [177]. ILK also participates in EMT stimulated by TGF-β by coupling this cytokine to its activation of AKT [178], and to its elevated expression of MMP-2 and uPA [91]. Collectively, these findings suggest that ILK may function analogously to FAK in mediating oncogenic signaling by TGF-β, and as such, suggest that ILK interfaces integrin signaling with that stimulated by TGF-β in epithelial cells undergoing EMT.

NF-κB

NF-κB is a principal player involved in regulating the production of proinflammatory cytokines [179], and in stimulating tumor growth, vascularization, survival and invasion [179]. In addition, NF-κB activity was observed to be essential in mediating the ability of Ras-transformed breast cancer cells to undergo EMT and colonize the lung when stimulated by TGF-β [48,180]. Along these lines, NF-κB activity also associates with several hallmarks of EMT, including downregulated E-cadherin expression and upregulated expression of vimentin [181]. It is interesting to note that TGF-β typically represses NF-κB activity in normal epithelial cells [47,182,183], but readily induces the activation of this transcription factor in their malignant counterparts [47,183]. Recently, we demonstrated that the activation of NF-κB by TGF-β transpires via the aberrant formation of a TAB1:xIAP:TAK1:IKKβ signaling module that only materializes in malignant MECs, or in normal MECs following their induction of EMT by TGF-β [47]. Functionally, the formation of TAB1:xIAP:TAK1:IKKβ complexes is essential for TGF-β stimulation of Cox-2 expression and its induction of EMT and invasion in normal and malignant MECs [47,183], as well as mammary tumor growth in immunocompetent and immunocompromised mice [47], suggesting a potentially important role of NF-κB in regulating innate immunity by TGF-β. Collectively, these findings demonstrate the role of NF-κB in supporting the development of oncogenic signaling by TGF-β in normal and malignant cells, particularly its ability to drive the growth, metastasis and EMT of tumors in response to TGF-β.

MAP kinases

Members of the MAP kinase family of protein kinases, which includes ERK1/2, JNKs and p38 MAPKs, have all been implicated in mediating EMT and metastasis stimulated by TGF-β [39,184,185]. For instance, pharmacological inhibition of ERK1/2 in MECs uncouples TGF-β from inducing EMT and its associated formation of stress fibers and delocalization of ZO-1 and E-cadherin [184]. Similarly, inactivation of JNK also prevents the ability of TGF-β to stimulate the morphological and transcriptional changes that drive EMT in epithelial cells [93,162]. Indeed, the activation of JNK by TGF-β induces fibronectin expression during EMT, and during fibroproliferative disorders that may progress to carcinoma [186]. Along these lines, collagen I and other ECM proteins can promote EMT via their activation of PI3K, Rac1 and JNK [187]; however, while it remains to be determined whether TGF-β is intimately involved in this ECM-dependent induction of EMT, it seems likely that the ability of TGF-β to stimulate the synthesis and secretion of ECM components is reciprocated by the ability of the ECM to establish paracrine and autocrine TGF-β signaling loops that perpetuate EMT in normal and malignant epithelial cells.

Besides its ability to activate ERK1/2 and JNK, TGF-β also stimulates p38 MAPK during its induction of EMT in normal and malignant cells [39]. Interestingly, the activation of p38 MAPK by TGF-β requires the expression and activity of either β1 or β3 integrins [39,43,44]. Indeed, we established the necessity of β3 integrin to form oncogenic signaling complexes with TβR-II, resulting in its phosphorylation on Y284 by Src [43,44]. Once phosphorylated, Y284 functions as a SH2-binding site that coordinates the recruitment of either ShcA or Grb2, as well as their subsequent activation of p38 MAPK [44]. Most importantly, pharmacologic or genetic inactivation of this oncogenic signaling axis prevented TGF-β from stimulating the growth and pulmonary metastasis of breast cancers produced in mice [45]. Finally, the activation of p38 MAPK not only induces EMT, but also stimulates the expression of prometastatic genes, particularly TβR-II and MMPs 2 and 9 [188,189], which collectively points to the importance of inappropriate p38 MAPK activation in mediating the conversion of TGF-β from a tumor suppressor to a tumor promoter.

Mechanisms of gene regulation by TGF-β

The importance of aberrant genetic and epigenetic events in promoting tumorigenesis is highlighted by the consistent and repeated finding that cancer cells that have lost their ability to regulate various rate-limiting steps that normally suppress malignant development. These untoward events typically are associated with:

- Mutational activation of oncogenes
- Mutational inactivation of tumor suppressor genes
- Amplified or silenced expression of genes coupled to the development of cancer hallmarks [190]

Although many of the signaling systems and genes targeted by TGF-β during its activation of EMT have been discussed above, the succeeding sections focus on the transcriptional mechanisms that orchestrate its transitioning of epithelial cells into their mesenchymal counterparts (Figure 3).

Nuclear factors

The Snail family of transcription factors are master regulators of EMT and include:

- SNAI1 (Snail) and SNAI2 (Slug)
- Two ZEB factors: ZEB1 and ZEB2 (SIP1)
- FOXC2 [110,191]

Indeed, the binding of Snail to conserved E-box sequences present in E-cadherin promoter is classically associated with EMT and the repression of E-cadherin expression, as well as that of the aforementioned cell polarity genes, occludin and claudin [192]. The essential function of various Snail family members in mediating EMT and cancer metastasis has been extensively reviewed, and as such, readers desiring a more in-depth description of their functions and behaviors in governing EMT are directed to several recent reviews [109,191]. Besides Snail family members, emerging evidence also implicates dysregulated Myc expression in promoting the ability of epithelial cells to undergo EMT in response to TGF-β. Indeed, the tumor-suppressing activity of TGF-β is intimately linked to its ability to rapidly repress Myc expression in epithelial cells [11,13]. Accordingly, uncoupling TGF-β from regulation of Myc expression is a common occurrence in developing carcinomas, resulting in their insensitivity to cytostasis mediated by TGF-β [193,194]. Somewhat unexpectedly, Myc was recently observed to function cooperatively with Smad4 to induce Snail expression during TGF-β stimulation of EMT in MECs [195]. Taken together, these findings suggest that Myc functions as a molecular detector that enables epithelial cells to sense TGF-β as a mediator of cytostasis or EMT.

STAT3

Signal transducer and activator of transcription 3 (STAT3) is a critical component of cell survival and proliferative responses, and its inappropriate activation can endow this transcription factor with oncogene-like properties in developing and progressing neoplasms [196]. A recent study has suggested that TGF-β couples to STAT3 phosphorylation and activation via a protein kinase A (PKA)-dependent mechanism [197]. Moreover, STAT3 activation by TGF-β is necessary for its ability to induce apoptosis and EMT [197], and to stimulate the invasion and metastasis of Smad4-deficient pancreatic cancer cells [198]. In addition, carcinoma cells that overexpressed epidermal growth factor receptors (EGFR) readily acquired EMT phenotypes when stimulated with epidermal growth factor (EGF), a cellular reaction that required EGF/EGFR to activate STAT3 and its subsequent upregulation of TWIST [199]. Thus, while several studies have shown EGF to cooperate with TGF-β in mediating tumorigenesis, the extent to which this tumor- and EMT-promoting alliance requires STAT3 remains to be determined definitively.

Estrogen receptor-α

Aberrant repression of the nuclear hormone receptor, estrogen receptor (ER)-α, has long been recognized as a major event that promotes the development and progression of mammary tumors, as well as significantly worsens the clinical prognosis of patients with metastatic breast cancer [200,201]. In addition to its prominent role in regulating mammary gland development and homeostasis, ER-α also prevents the ability of malignant MECs to acquire EMT and metastatic phenotypes, doing so via its stimulation of metastasis tumor antigen 3 (MTA3) expression, which in turn represses the expression of Snail [202]. Thus, inactivation or loss of ER-α in MECs promotes their EMT and invasion by allowing for their expression of Snail. Somewhat surprisingly, constitutive Snail expression in breast cancer cells was observed to inhibit ER-α expression [203], leading to enhanced invasion of these ER-α-deficient breast cancer cells. It is interesting to note that the physiological actions of estrogen in mammary tissues typically oppose those of TGF-β. Accordingly, inactivation of ER-α signaling led to elevated expression of components of the TGF-β signaling system and, presumably, to enhanced EMT in breast cancer cells [203]. Thus, Snail appears to function as a novel molecular sensor that integrates the opposing cellular functions of ER-α and TGF-β, particularly their ability to inhibit and stimulate EMT, respectively.

TGF-β, microRNAs & EMT

A number of recent studies have established microRNAs as important players that participate in cell and tissue development, as well as in controlling cell proliferation and motility through their ability to repress mRNA translation, or to induce mRNA degradation [204–207]. These studies have also shown that a single microRNA can repress the translation of multiple transcripts, and as such, dysregulated expression of a single microRNA, either positively or negatively, could initiate a cascade of gene silencing events capable of eliciting disease development in humans, including cancer. Accordingly, aberrant regulation of several microRNAs (or miRs) is observed in human cancers (see [208]), especially in those of

the breast, which can in fact be subtyped based on their differential expression of various microRNAs [206,209]. Along these lines, microRNAs also play a prominent role in regulating the expression of EMT-related genes. For instance, members of the miR-200 family suppress EMT by downregulating the expression of ZEB1 and ZEB2 (SIP1), which, as mentioned above, function in repressing the expression of E-cadherin [210–212]. Indeed, miR-200 family member expression marks epithelial cells that express E-cadherin and not vimentin, as well as identifies cancer cells that are poorly motile [213]. With respect to EMT and its regulation by TGF-β, a recent study established that this cytokine downregulates the expression of microRNA-200 family members and miR-205, which promotes ZEB1 and ZEB2 expression and their initiation of EMT [212]. In addition, these same microRNAs are frequently downregulated in invasive human breast cancer cells that exhibit a mesenchymal-like morphology [212]. Somewhat surprisingly, elevated ZEB1 expression also was found to repress that of miR-41 and miR-200c, both of which belong to the miR-200 family and whose absence establishes a negative feedback loop that stabilizes the acquisition of EMT phenotypes in epithelial cells [214].

In contrast to the miR-200 family of microRNAs, metastatic breast cancers were found to preferentially upregulate their expression of miR-10b, which promotes the invasion and metastasis of malignant MECs both *in vitro* and *in vivo* [215]. Mechanistically, Twist was observed to induce miR-10b expression that results in the diminished translation of HoxD10 transcripts, and also the induction of the prometastatic gene, *RhoC* [215]. More recently, administration of TGF-β to normal MECs induced miR-155 expression via a Smad4-dependent mechanism, an event that elicited EMT in cytokine-stimulated MECs [216]. Once expressed, miR-155 abrogated MEC expression of RhoA and prevented their ability to undergo EMT in response to TGF-β [216]. Similar overexpression of miR-21 is also observed in human cancers and results in the repression of the tumor suppressor, tropomyosin-1 [217,218]. The net effect of these events is the enhanced ability of breast cancer cells to grow in an anchorage-independent fashion [219], and to resist apoptotic stimuli in part via upregulated expression of the survival factor, Bcl-2 [217–219]. As above, the ability of TGF-β to induce EMT has been linked to its induction of miR-21 [220], which enhances cancer cell motility and invasive migration by downregulating tropomyosin expression [221–223].

Taken together, these findings suggest that the ability of TGF-β to govern microRNA expression plays an important role in dictating whether this cytokine propagates tumor-suppressing or -promoting signals to responsive cells; they also suggest that the development of chemotherapeutic agents capable of targeting microRNAs may function in 'normalizing' carcinoma cells and, consequently, rendering them insensitive to the oncogenic activities of TGF-β.

DNA hypermethylation

DNA hypermethylation is well established in its ability to aberrantly silence the expression of tumor suppressor genes in developing and progressing carcinomas [107]. Importantly, epigenetic silencing of the E-cadherin promoter via hypermethylation leads to morphological and differential gene-expression profiles indicative of EMT phenotypes [107,224]. Besides silencing of the E-cadherin promoter, EMT and mammary tumorigenesis usurp the inactivation of p16INK4a as a means to promote expanded DNA hypermethylation. Indeed, Roberts *et al.* [225] observed the loss of p16INH4a expression to depress that of the polycomb genes, *EZH2* and *SUZ12*, which collectively enhance DNA hypermethylation and the generation of MECs locked into a perpetual plastic state. Interestingly, the repression of E-cadherin expression during EMT appears to function as a prerequisite for directed gene hypermethylation during the development and progression of mammary tumorigenesis [226]. Moreover, hypermethylation of the E-cadherin promoter served to mark stable EMT in Ras-transformed MECs that was induced by serum versus a transient EMT induced in these same MECs by TGF-β [226]. Clearly, additional investigations are warranted to further our understanding of the linkages between TGF-β and DNA hypermethylation in mediating EMT in normal and malignant cells. Indeed, upregulated ZEB1 expression and its ability to induce EMT is tightly correlated with the loss of E-cadherin expression in cultured epithelial cells, and in metastatic carcinoma cells *in vivo* [227]. Based on these findings, it is tempting to speculate that initiation of EMT results in the expression of Snail family members that collectively function in repressing that of E-cadherin, as well as the subsequent recruitment of DNA methyltransferases that potentiate and stabilize the EMT phenotype.

Conclusion & future perspective

Embryogenesis and its associated EMT creates progenitor cells that ultimately give rise to

Executive summary

Defining the epithelial–mesenchymal transition

- Epithelial–mesenchymal transition (EMT) is defined by the morphologic and genetic transition of epithelial cells to fibroblastoid- or mesenchymal-like cells.
- The major cell–cell junctions include tight junctions, adherens junctions and desmosomes.
- Tight junctions are composed of claudins, occludins and junction-adhesion-molecules (JAMs), which are linked to the actin cytoskeleton via ZO-1, -2 and -3.
- During EMT, Par6 recruits the E3 ubiquitin ligase, Smurf1, which ubiquitinates RhoA, leading to its degradation and dissolution of tight junctions.
- Adherens junctions consist of E-cadherin that is linked to the actin cytoskeleton by α- and β-catenins.
- EMT represses E-cadherin transcription and disrupts its localization at the plasma membrane.

EMT, TGF-β & cell microenvironments

- Tumors house a mixture of malignant and normal cells, including fibroblasts, endothelial and infiltrating immune cells, which collectively comprise the tumor microenvironment.
- Transient disruption of the extracellular matrix (ECM) and epithelial cell microenvironments are characteristic of physiological EMT. By contrast, chronic disruptions within carcinoma cell microenvironments elicits pathologic EMT and its ability to support cancer cell invasion and metastasis.
- Matrix metalloproteinases (MMPs) comprise a large family of proteases that regulate essential steps of embryogenesis and tissue morphogenesis, wound healing and cell growth. MMPs also degrade nearly all ECM and basement membrane components, leading to the development and progression of human malignancies.
- Neural cell adhesion molecule (NCAM) is a member of the immunoglobulin superfamily whose expression is increased during EMT and is cleaved by MMP-28.
- Urokinase plasminogen activator (uPA) is a serine protease whose expression is elevated during EMT and associates with advanced disease states and poor clinical outcomes.
- Plasminogen activator inhibitor (PAI)-1 antagonizes tissue plasminogen activator (tPA) and uPA; its expression is also increased during EMT and associates with advanced disease states and poor clinical outcomes.
- EMT leads to the upregulation of collagen and fibronectin, whose expression drastically alters cell microenvironments.
- Vimentin is an intermediate filament protein, while α-smooth muscle actin is a component of contractile microfilaments. Upregulated expression of both proteins are considered to be markers of fully transitioned cells.

Transmembrane & membrane proximal protein complexes that impact TGF-β signaling & EMT

- α- and β-integrin heterodimers function in linking the ECM to intracellular signaling pathways, and to the cellular cytoskeletal system.
- Integrins interact with several intracellular kinases, as well as several transmembrane receptor tyrosine kinases (RTKs).
- Integrin β1 and β3 interact with TβR-II and profoundly affect downstream signaling events stimulated by TGF-β.
- β3 integrin is upregulated dramatically during EMT induced by TGF-β.
- Interaction between αvβ3 integrin and TβR-II leads to Src-mediated phosphorylation of TβR-II at Tyr284, which binds Grb2 and promotes the activation of downstream mitogen-activated protein kinases (MAPKs).
- TGF-β stimulates cancer progression and metastasis by cooperating with integrins.
- Focal adhesion kinase (FAK) is required for EMT stimulated by TGF-β.
- p130Cas inhibits Smad3 activity and alters cytostasis induced by TGF-β.
- Hic5 is a member of the paxillin superfamily that is induced by and required for EMT stimulated by TGF-β.
- TRAF6 interacts physically with both TβR-I and TβR-II, leading to TGF-β-activated kinase (TAK)1 activation and the stimulation of p38 MAPK and c-Jun N-terminal kinase (JNK).

Signaling systems involved in EMT stimulated by TGF-β

- TGF-β activates Smad2/3, which play important roles during TGF-β stimulation of cancer cell EMT and metastasis.
- Small GTPases RhoA, Rac1 and Cdc42 regulate the formation of stress fibers, lamellipodia or filopodia, respectively, and are intimately involved in EMT stimulated by TGF-β.
- β1 and β3 integrins activate the Ser/Thr protein kinase ILK, which stimulates MAP kinases, PI3K/AKT and small GTPases, and inhibits of GSK3β.
- ILK participates in EMT stimulated by TGF-β by coupling this cytokine to AKT activation, and MMP-2 and uPA expression.
- NF-κB activity enables Ras-transformed breast cancer cells to undergo EMT and colonize the lung when stimulated by TGF-β.
- MAP kinase family members, including ERK1/2, JNK and p38 MAPK mediate EMT and metastasis stimulated by TGF-β.

Mechanisms of gene regulation by TGF-β

- Snail transcription factor family members are master regulators of EMT and include SNAI1 (Snail) and SNAI2 (Slug); ZEB1 and ZEB2 (SIP1); and FOXC2.

Executive summary (cont.)

Mechanisms of gene regulation by TGF-β (cont.)

- Dysregulated Myc expression promotes EMT in response to TGF-β, while the tumor-suppressing activity of TGF-β is intimately linked to its repression of Myc expression in epithelial cells.
- STAT3 mediates cell survival and proliferative signals, and serves as an oncogene in several human cancers.
- TGF-β activates STAT3 *via* a protein kinase A (PKA)-dependent mechanism, leading to the induction of EMT.
- ER-α promotes mammary gland develop and homeostasis, and suppresses EMT by inducing the expression of metastasis tumor antigen (MTA)3, which represses the expression of Snail.
- microRNAs are essential mediators of all facets of cell and tissue development, and of cell proliferation, motility and survival.
- Members of the miR-200 family suppress EMT by downregulating the expression of ZEB1 and ZEB2.
- Epigenetic silencing of the E-cadherin promoter via hypermethylation promotes the acquisition of EMT phenotypes and gene-expression profiles.
- EMT and mammary tumorigenesis usurp the inactivation of p16INK4a as a means to expand aberrant DNA hypermethylation.

Redefining EMT induced by TGF-β

- Inappropriate reactivation of EMT by TGF-β in malignant tissues promotes the selection and expansion of cancer stem and progenitor cells.
- Targeting the molecular links between TGF-β, EMT and stemness reduces breast cancer tumorigenicity.
- The development of pharmacological agents that inhibit EMT stimulated by TGF-β may provide new avenues to manipulate the behaviors of normal and cancer stem cells, and to alleviate the acquisition of cancer metastasis.

every cell and tissue type within mature organisms. For instance, EMT underlying gastrulation results in the generation of the mesoderm, which subsequently develops along distinct differentiation pathways that elicit the production of muscle, bone and connective tissues [7]. Similarly, a single mammary stem cell can give rise to both the outer myoepithelial and inner luminal layers that comprise the branched structure of these glands [228–230]. These and other studies suggest an important link between physiologic EMT and the generation and maintenance of stem cells, of which both phenomena require signaling inputs elicited by the TGF-β signaling system [231]. Given the parallels between physiologic and pathophysiologic EMT, it is fitting to find that the inappropriate reactivation of EMT in malignant tissues also promotes the selection and expansion of cancer stem cells. For instance, aggressive and poorly differentiated breast cancer and glioma cells exhibit gene signatures characteristic of stem cells [232]. In addition, TGF-β stimulation of EMT in human and mouse MECs established a population of transitioning cells that possessed stem cell-like properties [233,234], suggesting that EMT induced by TGF-β promotes 'stemness'. Along these lines, inactivation of TGF-β signaling in cancer stem cells induced a mesenchymal–epithelial transition that reestablished a more epithelial-like morphology in aggressive cancer cells [235]. Thus, these intriguing findings suggest that the ability of TGF-β to stimulate the selection and expansion of stem cell-like progenitors in post-EMT epithelial cells may represent the molecular crux that endows TGF-β with oncogenic activity. Clinically, these findings also suggest that the development of chemoresistance may reflect the induction of EMT and its expansion of cancer stem cells by TGF-β. If correct, then the studies reviewed herein offer important insights into how science and medicine may one day target the TGF-β signaling system and its coupling to EMT in order to regulate the behaviors and activities of normal and cancer stem cells, and alleviate the devastating effects of TGF-β in promoting the acquisition of invasive and metastatic phenotypes in human cancers.

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The TGF-β paradox in human cancer: an update

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TGF-β plays an essential role in maintaining tissue homeostasis through its ability to induce cell cycle arrest, differentiation and apoptosis, and to preserve genomic stability. Thus, TGF-β is a potent anticancer agent that prohibits the uncontrolled proliferation of epithelial, endothelial and hematopoietic cells. Interestingly, tumorigenesis typically elicits aberrations in the TGF-β signaling pathway that engenders resistance to the cytostatic activities of TGF-β, thereby enhancing the development and progression of human malignancies. Moreover, these genetic and epigenetic events conspire to convert TGF-β from a suppressor of tumor formation to a promoter of their growth, invasion and metastasis. The dichotomous nature of TGF-β during tumorigenesis is known as the 'TGF-β paradox', which remains the most critical and mysterious question concerning the physiopathological role of this multifunctional cytokine. Here we review recent findings that directly impact our understanding of the TGF-β paradox and discuss their importance to targeting the oncogenic activities of TGF-β in developing and progressing neoplasms.

TGF-β & the tumor microenvironment

TGF-β & fibroblasts

Tumor development in many respects mirrors that of an organ, albeit in a highly dysfunctional and disorganized manner. For instance, whereas normal tissue specification requires reciprocal signaling inputs from distinct cell types and matrix proteins, the phenotype of developing carcinomas is similarly dictated by the dynamic interplay between malignant cells and their accompanying stroma, which houses fibroblasts and endothelial cells (ECs), as well as a variety of infiltrating immune and progenitor cells [1,2]. Moreover, tumor-reactive stroma not only plays an important role during cancer initiation and progression, but also in determining whether TGF-β suppresses or promotes tumor formation (Figure 1) [3–5]. Along these lines, TGF-β exerts its anti-tumor activities by regulating epithelial cell behavior, and by regulating that of adjacent fibroblasts, which synthesize and secrete a variety of cytokines, growth factors and extracellular matrix (ECM) proteins that mediate tissue homeostasis and suppress cancer development. Thus, inactivating paracrine TGF-β signaling between adjacent epithelial and stromal compartments promotes cellular transformation, as well as induces the growth, survival and motility of developing neoplasms [6,7]. For instance, rendering fibroblasts deficient in the expression of the TGF-β type II receptor (TβR-II), which manifests as insensitivity to TGF-β, results in the formation of prostate intraepithelial neoplasia and invasive carcinoma of the forestomach [3]. Conditional deletion of TβR-II in mammary gland fibroblasts enhanced their proliferation and abundance within abnormally developed ductal units [8]. Interestingly, grafting a mixture of TβR-II-deficient mammary fibroblasts with mammary carcinoma cells under the subrenal capsule significantly enhanced the growth and invasion of breast cancer cells. The enhanced tumorigenicity of implanted mammary carcinoma cells was not recapitulated in grafts containing TGF-β-responsive fibroblasts, which failed to synthesize and secrete the high levels of TGF-α, macrophage-stimulating protein (MSP) and hepatocyte growth factor (HGF) produced by their TβR-II-deficient counterparts [3–5,8]. Thus, TGF-β signaling in fibroblasts suppresses their activation of cancer-promoting paracrine signaling axes that target adjacent epithelial cells. Somewhat surprisingly, TβR-II-deletion in mammary carcinoma cells resulted in the activation of two tumorigenic paracrine signaling axes comprised of SDF-1:CXCR4 and CXCL5:CXCR2, which collectively function in recruiting immature $GR1^+CD11b^+$ myeloid cells to developing mammary tumors [9]. Upon their arrival within mammary tumor microenvironments, $GR1^+CD11b^+$ cells promote breast cancer cell invasion and metastasis by attenuating host tumor immunosurveillance and by stimulating MMP expression [9]. Recently, the ability of TGF-β to induce cell cycle progression in glioma cells required initiation of

Keywords
angiogenesis ■ cancer ■ cell invasion ■ epithelial–mesenchymal transition ■ metastasis ■ signal transduction ■ transforming growth factor-β

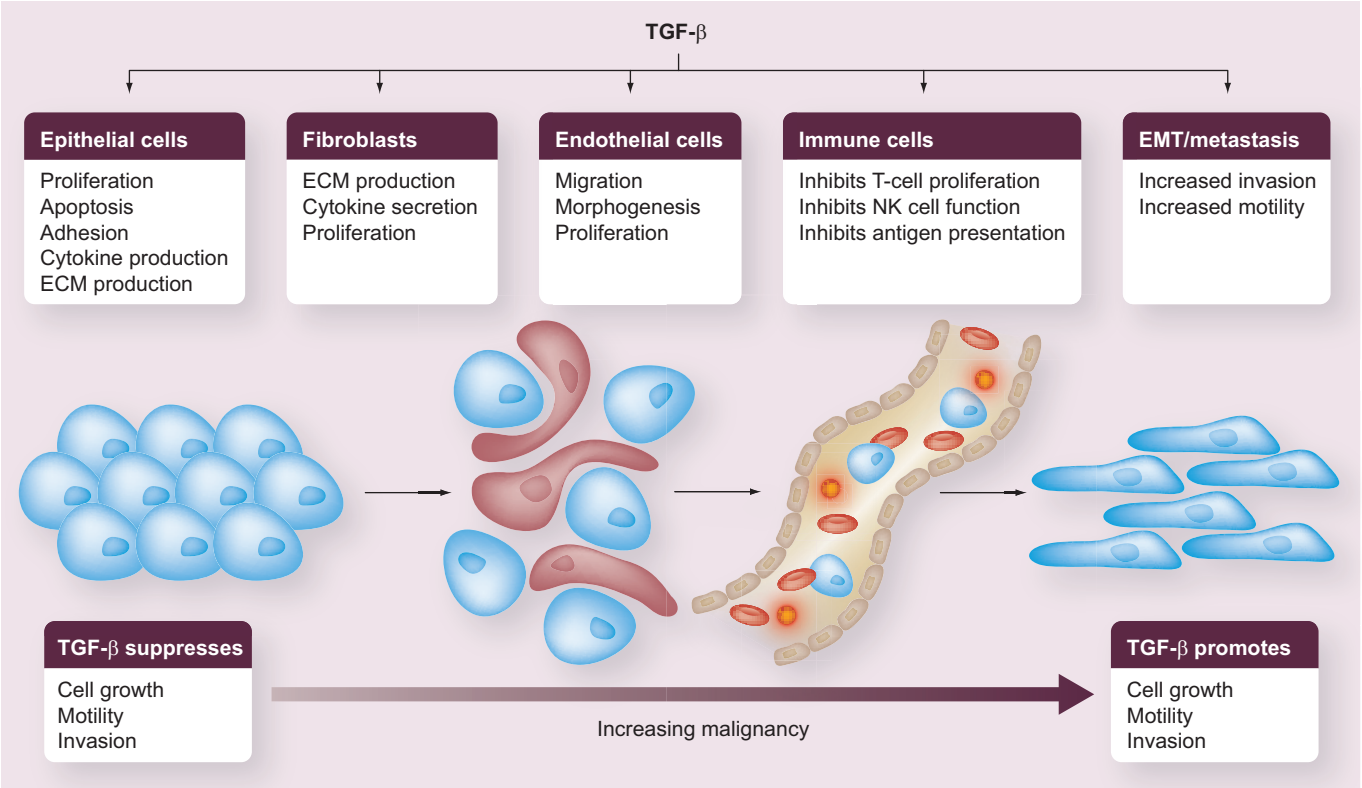

Figure 1. Cellular targets of TGF-β during the development and progression of human cancers. TGF-β is a multifunctional cytokine that normally suppresses cell proliferation, differentiation and apoptosis, as well as regulating cell and tissue homeostasis. Under normal physiological conditions, TGF-β functions as a tumor suppressor by preventing the ability of cells to progress through the cell cycle, or by stimulating the ability of cells to undergo apoptosis or differentiation. However, genetic and epigenetic events that transpire during tumorigenesis can convert TGF-β from a tumor suppressor to a tumor promoter, particularly the ability of cancer cells to acquire invasive and metastatic phenotypes. The oncogenic activities of TGF-β are coordinated by dysregulated autocrine and paracrine signaling networks that take place between epithelial (blue), fibroblasts (maroon), endothelial (yellow) and immune cells (orange), that collectively promote tumor angiogenesis, invasion and metastasis, and inhibit most immunosurveillance within tumor microenvironments. See text for specific examples of how TGF-β signaling becomes dysregulated during tumorigenesis.
ECM: Extracellular matrix; NK: Natural killer.

autocrine PDGF-B signaling. Importantly, the proliferation-promoting properties of TGF-β and Smad2/3 only occurred in glioma lacking methylation of the *PDGF-B* gene, suggesting that the methylation status of *PDGF-B* determines the oncogenic activities of TGF-β, in part via autocrine PDGF-B signaling within tumor microenvironments [10].

Tumorigenesis is often accompanied by intense desmoplastic and fibrotic reactions, which elicit the formation of rigid tumor microenvironments that enhance the selection and expansion of metastatic cells [11,12]. Lysyl oxidases (LOXs) belong to a five-gene family of copper-dependent amine oxidases (i.e., LOX, LOXL, LOXL2, LOXL3 and LOXL4) that function in cross-linking collagens to elastin in the ECM [13,14]. Mechanistically, the activation of these cross-linking reactions by LOXs secreted by fibroblasts and epithelial cells serves to increase the tensile strength and structural integrity of tissues during embryonic development and organogenesis, as well as during the maintenance of normal tissue homeostasis [13,14]. Similar to TGF-β, members of the LOX family have been associated with tumor suppression and tumor promotion. Indeed, the transformation of fibroblasts by oncogenic Ras is suppressed by LOX and its ability to bind, oxidize and inactivate growth factors housed in cell microenvironments, which presumably contributes the loss of cyclin D1 expression observed in LOX-expressing fibroblasts [15,16]. More recently, LOX was observed to interact physically with TGF-β1 and alter its ability to stimulate Smad3 in cultured osteoblasts [17], while LOXL4 expression inhibited TGF-β stimulation of liver cancer cell invasion through synthetic basement membranes [18]. Thus, these findings implicate LOXs as potential suppressive agents within tumor microenvironments. In stark contrast, aberrant LOX activity also is associated with cancer progression, particularly the selection, expansion

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p130Cas Is Required for Mammary Tumor Growth and Transforming Growth Factor-β-mediated Metastasis through Regulation of Smad2/3 Activity*[S]

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During breast cancer progression, transforming growth factor-β (TGF-β) switches from a tumor suppressor to a pro-metastatic molecule. Several recent studies suggest that this conversion in TGF-β function depends upon fundamental changes in the TGF-β signaling system. We show here that these changes in TGF-β signaling are concomitant with aberrant expression of the focal adhesion protein, p130Cas. Indeed, elevating expression of either the full-length (FL) or just the carboxyl terminus (CT) of p130Cas in mammary epithelial cells (MECs) diminished the ability of TGF-β1 to activate Smad2/3, but increased its coupling to p38 MAPK. This shift in TGF-β signaling evoked (i) resistance to TGF-β-induced growth arrest, and (ii) acinar filling upon three-dimensional organotypic cultures of p130Cas-FL or -CT expressing MECs. Furthermore, rendering metastatic MECs deficient in p130Cas enhanced TGF-β-stimulated Smad2/3 activity, which restored TGF-β-induced growth inhibition both *in vitro* and in mammary tumors produced in mice. Additionally, whereas elevating TβR-II expression in metastatic MECs had no affect on their phosphorylation of Smad2/3, this event markedly enhanced their activation of p38 MAPK, leading to increased MEC invasion and metastasis. Importantly, depleting p130Cas expression in TβR-II-expressing metastatic MECs significantly increased their activation of Smad2/3, which (i) reestablished the physiologic balance between canonical and noncanonical TGF-β signaling, and (ii) reversed cellular invasion and early mammary tumor cell dissemination stimulated by TGF-β. Collectively, our findings identify p130Cas as a molecular rheostat that regulates the delicate balance between canonical and noncanonical TGF-β signaling, a balance that is critical to maintaining the tumor suppressor function of TGF-β during breast cancer progression.

Invasion and metastasis are the most lethal characteristics of breast cancer (1, 2). Transforming growth factor-β (TGF-β)[2] is a powerful suppressor of mammary tumorigenesis, doing so through its ability to repress mammary epithelial cell (MEC) proliferation, as well as through its creation of cell microenvironments that inhibit MEC motility, invasion, and metastasis (2). During breast cancer progression, the tumor suppressing function of TGF-β is frequently subverted, thus transforming TGF-β from a suppressor of breast cancer formation to a promoter of its growth and metastasis (2–4). Unfortunately, how mammary tumorigenesis overcomes the cytostatic function of TGF-β remains incompletely understood, as does the manner in which developing breast cancers ultimately sense TGF-β as a pro-metastatic factor.

Transmembrane signaling by TGF-β commences upon binding to its type II receptor (TβR-II), which recruits and activates its type I receptor (TβR-I), which then phosphorylates and activates Smads 2 and 3. Following their activation, Smads 2 and 3 form heteromeric complexes with Smad4, which collectively translocate to the nucleus to regulate a multitude of transcriptional events and cellular responses (*i.e.* apoptosis, cytostasis, and homeostasis, (5, 6)). In addition to stimulating Smad2/3, TGF-β also activates several noncanonical signaling systems, including members of the MAP kinase family (*e.g.* ERK1/2, JNK, and p38 MAPK (7)). Interestingly, several studies suggest that genetic and epigenetic events cooperate with aberrant Smad2/3 activities and functions to facilitate the conversion of TGF-β from tumor suppressor to a tumor promoter (8, 9). However, these and other studies also present strong evidence implicating dysregulated activation of several noncanonical TGF-β effectors during this same switch in TGF-β function (10). Thus, deciphering the relative contribution of signaling imbalances that arise between Smad2/3-dependent and -independent TGF-β signaling systems is essential to enhancing our understanding of how TGF-β ultimately promotes the development and progression of mammary tumorigenesis.

Recently, we identified a critical αvβ3 integrin:pY284-TβR-II:Grb2 signaling axis that mediates TGF-β stimulation of MAP kinases in normal and malignant MECs, leading to their acquisition of epithelial-mesenchymal transition, invasive, and metastatic phenotypes both *in vitro* and *in vivo* (11–13). Moreover, activation of this oncogenic signaling axis by TGF-β requires

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[2] The abbreviations used are: TGF-β, transforming growth factor-β; FAK, focal adhesion kinase; MEC, mammary epithelial cell; TβR-I, TGF-β type I receptor; TβR-II, TGF-β type II receptor; p130Cas, Crk-associated substrate; PAI-1, plasminogen activator inhibitor-1; NmuMG, normal murine mammary epithelial; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CMV, cytomegalovirus; shRNA, short hairpin RNA; β-gal, β-galactosidase; FL, full-length; CT, carboxyl-terminal.

β3 integrin to form complexes with TβR-II (11–13). Unfortunately, it remains uncertain as to whether this interaction is direct or facilitated through another scaffolding protein. As such, we sought to identify members of focal adhesion complexes as potential integrin effectors capable of contributing to altered TGF-β signaling.

p130Cas (Crk-associated substrate) functions as a molecular scaffold within focal adhesion complexes, and is readily phosphorylated by focal adhesion kinase (FAK) and Src (14). Additionally, p130Cas binds stably to a variety of signaling molecules, including the (i) protein-tyrosine kinases FAK, PYK2, Src, Fyn, and Abl; (ii) adaptor molecules Crk, CrkL, Trip6, and AJUBA; (iii) guanine nucleotide exchange factors AND34 and CG3; and (iv) the MAPK family member, JNK (15, 16). The extensive interactome of p130Cas ideally positions and enables this molecule to interpret and integrate a variety of signaling inputs arising from numerous receptor systems. Indeed, the biological importance of p130Cas is emphasized by studies showing that its genetic ablation in mice elicits embryonic lethality, whereas fibroblasts derived from p130Cas-deficient embryos exhibit drastically altered cytoskeletal architectures (17). Moreover, fibroblasts transformed by Src become significantly more invasive when engineered to simultaneously overexpress p130Cas (15). Patients with primary breast tumors expressing high levels of p130Cas (also known as breast cancer resistance-1) experience a more rapid disease recurrence and have a greater risk of resistance to tamoxifen therapy (18). Recent studies also indicate that specific overexpression of p130Cas/breast cancer resistance-1 expression can confer breast cancer resistance to adriamycin (19). Moreover, directed overexpression of p130Case in murine MECs significantly increased their proliferative and survival indices *in vivo*, as well as greatly reduced the latency of mammary tumors arising from murine mammary tumor virus-driven Her2/Neu expression in mice (20). This study also observed the expression of p130Cas to be up-regulated significantly in a subset of human breast cancer samples (20). Collectively, these findings highlight the critical roles played by p130Cas in regulating normal tissue morphogenesis, and in promoting breast cancer progression. With respect to TGF-β, a recent study identified p130Cas as a potential inhibitor of Smad3 function (21). However, the pathophysiological importance of this event, if any, in mediating the oncogenic activities of TGF-β and/or p130Cas during breast cancer progression remains to be established.

The objective of the present study was to determine the role of p130Cas in facilitating the acquisition of oncogenic signaling by TGF-β during breast progression. We show here that p130Cas expression is up-regulated significantly in metastatic breast cancer cells (murine 4T1/human MCF10A-Ca1a) as compared with their nonmetastatic counterparts (murine 67NR/human MCF10A). Moreover, increased p130Cas expression was consistent with a decrease in TGF-β1-induced Smad2/3 signaling. Indeed, overexpression of p130Cas in nonmetastatic MECs led to a decrease in Smad2/3 activity, whereas depletion of p130Cas in metastatic MECs increased Smad2/3 activity. Most importantly, we show for the first time that p130Cas is essential for TGF-β stimulation of breast cancer growth, invasion, and pulmonary dissemination in mice. Taken together, our findings establish p130Cas as a novel molecular rheostat that regulates the balance between canonical and noncanonical TGF-β signaling in developing mammary tumors, whose acquisition of metastatic phenotypes is potentiated by elevated p130Cas expression and its consequential disruption of homeostatic TGF-β signaling.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Normal murine NMuMG and metastatic 4T1 cells were obtained from ATCC (Manassas, VA) and cultured as described previously (12). 4T1 cells were engineered to stably express firefly luciferase by transfection with pNifty-CMV-luciferase (22), followed by their selection and isolation with Zeocin (500 μg/ml; Invitrogen). The creation of 4T1 cells lacking p130Cas was accomplished by their transduction with lentiviral particles encoding either a scrambled (*i.e.* non-silencing shRNA) or murine-directed p130Cas shRNA (pLKO.1; Thermo Scientific, Huntsville, AL). The production of pLKO.1 lentiviral particles and their transduction into target cells was accomplished as described previously (23). In addition, NMuMG and 4T1 cells also were transduced with murine ecotropic retroviral particles that encoded for full-length p130Cas (pMSCV-Cas-FL), the carboxyl terminus of p130Cas (amino acids 544–874) (pMSCV-Cas-CT), or TβR-II (pMSCV-TβR-II), and the resulting polyclonal populations were selected by yellow fluorescence protein, or hygromycin resistance (200 μg/ml).

Cell Proliferation Assays—NMuMG and 4T1 cells were seeded in 96-well plates (10,000 cells/well) and allowed to adhere for 4 h, whereupon varying concentrations of TGF-β1 (0–5 ng/ml) were administered. Agonist stimulations were allowed to proceed for 48 h at 37 °C and cellular DNA was radiolabeled by inclusion of [^{3}H]thymidine (1 μCi/well) during the final 6 h of TGF-β1 treatment. Afterward, the amount of [^{3}H]thymidine incorporated into cellular DNA was quantified by scintillation counting.

Immunoblot Assays—NMuMG and 4T1 cells were lysed on ice in three-dimensional RIPA buffer (50 mM Tris, 150 mM NaCl, 0.25% (v/v) sodium deoxycholate, 0.1% SDS (v/v), pH 7.4) supplemented with (i) protease inhibitor mixture (Sigma), and (ii) the phosphatase inhibitors sodium orthovanadate (10 mM), β-glycerophosphate (40 mM), and sodium fluoride (20 mM). Afterward, the resulting whole cell extracts were clarified by microcentrifugation prior to being immunoblotted with the following primary antibodies (dilution): (*a*) anti-phospho-p38 MAPK (1:500; Cell Signaling, Danvers, MA); (*b*) anti-p38 MAPK (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); (*c*) anti-phospho-Smad2 (1:1000; Cell Signaling); (*d*) anti-phospho-Smad3 (1:500; Cell Signaling); (*e*) anti-Smad 2/3 (1:1000; BD Biosciences); (*f*) anti-FAK (1:1000; Santa Cruz Biotechnology); (*g*) anti-p130Cas (1:1000; BD Biosciences); (*h*) phospho-p130Cas (1:1000; Cell Signaling); (*i*) anti-actin (1:1000; Santa Cruz Biotechnology); (*j*) lamin A/C (1:1000; Santa Cruz Biotechnology); and (*k*) E-Cadherin (1:2000; BD Biosciences).

Real-time PCR Analyses—Quiescent 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h in the absence or presence of the p38 MAPK inhibitor SB208530 (10 μM) and total RNA was isolated using the RNeasy Plus Kit (Qiagen, Valen-

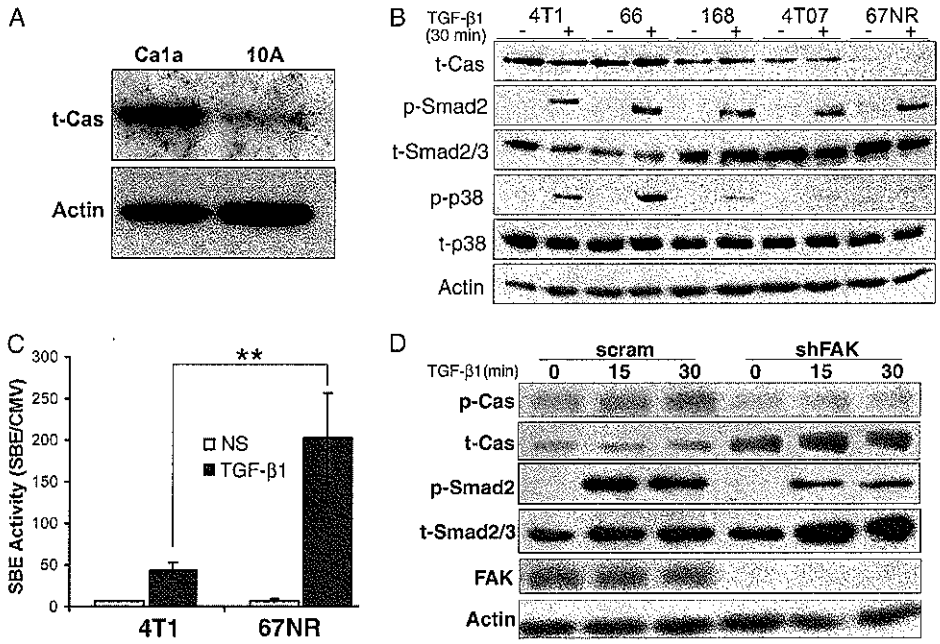

FIGURE 1. **Elevated p130Cas inhibits TGF-β-mediated Smad2/3 activation.** *A*, normal human MECs (*MCF-10A*) and their metastatic derivatives (*Ca1a*) were immunoblotted for p130Cas expression. β-Actin (*Actin*) is shown as loading control. Data are representative images from a representative experiment that was performed two times with identical results. *B*, murine breast cancer cells derived from the same primary Balb/c tumor, including the highly metastatic 4T1 and 66c14 (*66*) cells, the partially metastatic 168Farn (*168*) and 4T07 cells, and the nonmetastatic 67NR cells were immunoblotted for p130Cas expression, and the phosphorylation of Smad2 (*p-Smad2*) and p38 MAPK (*p-p38*) in response to TGF-β1 stimulation (5 ng/ml). Total Smad2/3 (*t-Smad2/3*), p38 MAPK (*t-p38*), and β-actin (*Actin*) were analyzed as loading controls. Data are from a representative experiment that was performed two times with identical results. *C*, 4T1 and 67NR cells were transiently transfected with pSBE-luciferase and pCMV-β-gal plasmids, and subsequently stimulated with TGF-β1 (5 ng/ml) for 18 h prior to measuring luciferase and β-gal activities. *NS*, no stimulation. Data are the mean ± S.E. ($n = 3$) of luciferase/β-gal activity ratios. **, $p = 0.01$. *D*, quiescent 4T1 cells that expressed either a scrambled (*scram*) or FAK-specific (*shFAK*) shRNA were stimulated with TGF-β1 (5 ng/ml) for varying times, and subsequently immunoblotted with phospho-specific antibodies against p130Cas (*p-Cas*) and Smad2 (*p-Smad2*) as indicated. Membranes were stripped and reprobed with antibodies against p130Cas (*t-Cas*), Smad2/3 (*t-Smad2/3*), β-actin (*Actin*), and FAK as indicated. Data are from a representative experiment that was performed at least three times with similar results.

cia, CA). Afterward, total RNA was reverse transcribed using the iScript cDNA Synthesis System (Bio-Rad) and semi-quantitative real-time PCR was conducted for PAI-1 using iQ SYBR Green (Bio-Rad) according to the manufacturer's recommendations and as described previously (23). Differences in RNA concentrations were controlled by normalizing individual gene signals to their corresponding glyceraldehyde-3-phosphate dehydrogenase signal.

Cell Fractionation Studies—Unstimulated and TGF-β1 (5 ng/ml)-stimulated NMuMG cells were lysed on ice in Buffer C (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, and 0.004% Nonidet P-40, pH 7.9) supplemented with protease inhibitor mixture (Sigma). Afterward, the resulting whole cell extract was subjected to a single freeze-thaw cycle, followed by microcentrifugation to yield a clarified cytoplasmic fraction. The remaining pellet was resuspended in Buffer N (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, and 10% glycerol, pH 7.9) supplemented with protease inhibitor mixture, and shaken vigorously for 2 h at 4 °C. Afterward, this mixture was subjected to microcentrifugation to yield a clarified nuclear fraction.

Three-dimensional Culture Assays—NMuMG (1×10^4) and 4T1 (5×10^3) cells were diluted in complete medium supplemented with 5% Cultrex (R&D Systems, Minneapolis, MN), and subsequently seeded in 48-well plates on top of a Cultrex cushion. Where indicated, 4T1 cells were grown in the presence of TGF-β1 (5 ng/ml). The medium/Cultrex mixture was replaced at 7 days, and organoids were allowed to grow for a total length of 10 days, at which point they were monitored for hollowing by phase-contrast microscopy and quantified by three individuals who were blinded to the culture conditions. 4T1 acinar size was quantified using Image J software.

Cell Invasion Assays—The ability of TGF-β1 (5 ng/ml) to alter the invasion of 4T1 cells (50,000 cells/well) was analyzed using a modified Boyden Chamber assay as described previously (13).

Luciferase Reporter Gene Assays—NMuMG cells were transiently transfected overnight with LT1 liposomes (Mirus, Madison, WI) that contained 300 ng/well of pSBE-firefly luciferase (4X-CAGA) cDNA and 50 ng/well of pCMV-β-gal cDNA. Afterward, the cells were stimulated for 24 h with TGF-β1 (5 ng/ml), and subsequently harvested and assayed for firefly luciferase (Promega, Madison WI) and β-gal (Clontech, Mountain View, CA) activities. In addition, 4T1 cells that stably expressed firefly luciferase under control of the CMV promoter were similarly (i) transfected with 300 ng/well of pSBE-*Renilla* luciferase (4X-CAGA); (ii) stimulated with TGF-β1; and (iii) assayed for *Renilla* and firefly luciferase using the Dual-Glo Assay System as above (Promega).

Immunofluorescent Analyses—4T1 cells (25,000 cells/well) were allowed to adhere overnight to glass coverslips. Afterward, the cells were washed extensively in phosphate-buffered saline and immediately stimulated with TGF-β1 (5 ng/ml). Upon completion of agonist stimulation, the cells were (i) fixed in 4% paraformaldehyde; (ii) permeablized in 0.1% Triton X-100; (iii) stained with anti-Smad 2/3 antibodies (1:100; BD Biosciences); and (iv) visualized by addition of biotinylated anti-mouse antibodies (1:1000) in conjunction with the addition of rhodamine-conjugated streptavidin (1:2000).

Tumor Growth, in Vivo Bioluminescent Imaging, and Immunohistochemical Analyses—Control or various 4T1 derivatives engineered to stably express firefly luciferase were resuspended in sterile phosphate-buffered saline (50 μl) and injected orthotopically into the mammary fatpad (10,000 cells/injection) of 6-week-old female Balb/c mice (Jackson Laboratory, Bar Harbor, ME). Primary 4T1 tumor growth and metastasis development were assessed by (i) weekly bioluminescent imaging of

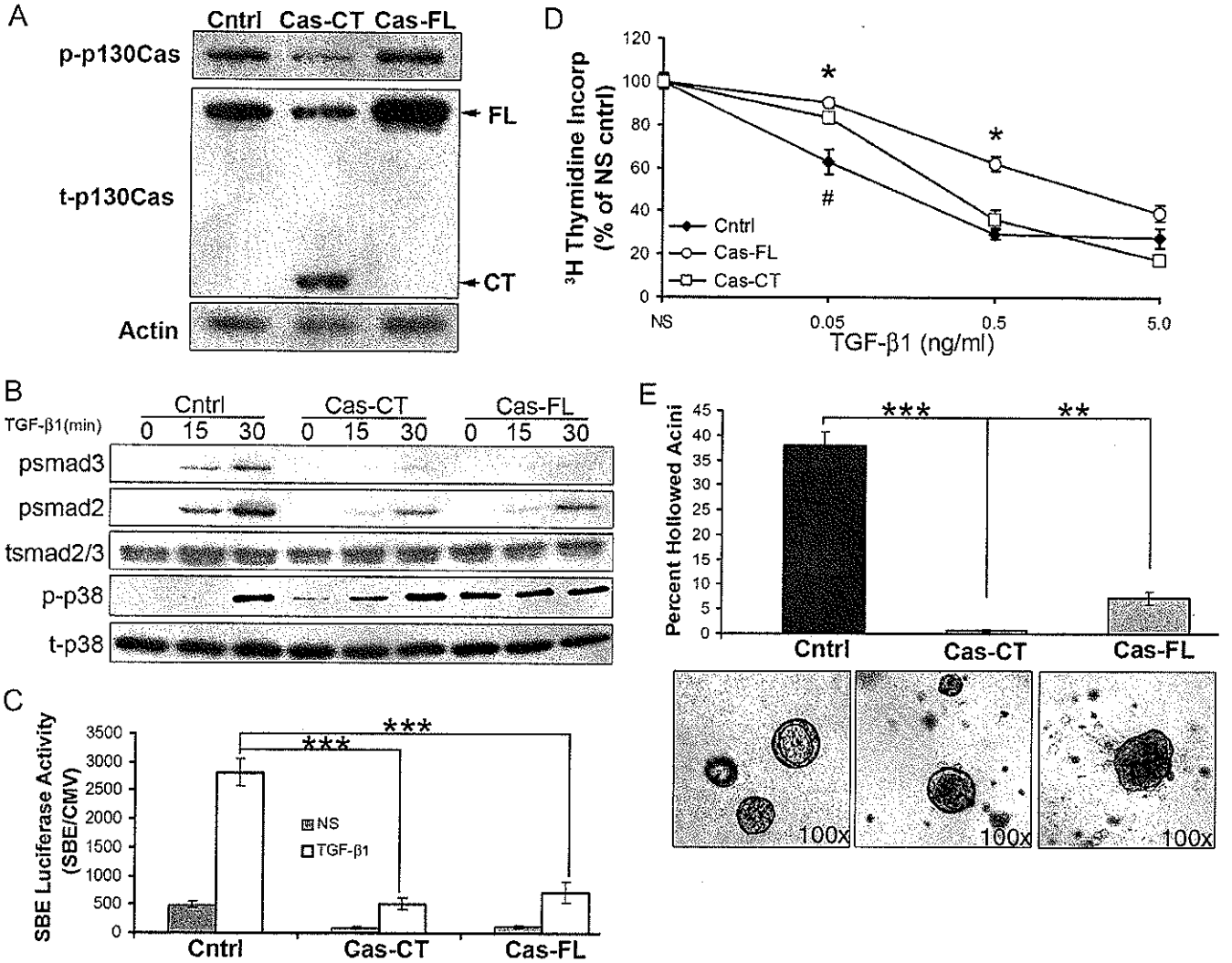

FIGURE 2. **Overexpression of p130Cas inhibits Smad2/3 activity and alters normal mammary epithelial acinar formation.** *A*, NMuMG cells were transduced with retroviral particles containing vector control (*cntrl*), full-length p130Cas (*Cas-FL*), or the carboxyl terminus of p130Cas (*Cas-CT*). Stable transgene expression was assessed by immunoblotting for phosphorylated (*p-p130Cas*) and total p130Cas (*t-p130Cas*). β-Actin (*Actin*) is shown as a loading control. *B*, the p130Cas-manipulated cell lines as described in *A* were stimulated with TGF-β1 (5 ng/ml) for the indicated times, and subsequently analyzed for the phosphorylation of Smad2 (*psmad2*), Smad3 (*psmad3*), and p38 MAPK (*p-p38*). Membranes were stripped and reprobed for total Smad2/3 (*tsmad2/3*) and p38 MAPK (*t-p38*) as loading controls. Data are from representative experiments that were performed at least three times with identical results. *C*, control (cntrl) and p130Cas-expressing (Cas-CT and Cas-FL) NMuMG cells were transiently co-transfected with pSBE-luciferase and pCMV-β-gal plasmids, and subsequently stimulated overnight with TGF-β1 (5 ng/ml) prior to measuring luciferase and β-gal activities. *NS*, no stimulation. Data are the mean ± S.E. of SBE/CMV activity ratios observed in three independent experiments completed in triplicate. ***, $p < 0.001$). *D*, control (*cntrl*) and p130Cas-expressing (*Cas-CT* and *Cas-FL*) NMuMG cells were stimulated with increasing concentrations of TGF-β1 (0–5 ng/ml) for 48 h, and subsequently assayed for [^{3}H]thymidine incorporation into cellular DNA. Data are the mean ± S.E. quantities of incorporated [^{3}H]thymidine normalized to unstimulated controls observed in three independent experiments completed in triplicate (*, $p < 0.05$ between Cntrl and Cas-FL; #, $p < 0.05$ between Cntrl and Cas-CT). *E*, the p130Cas-manipulated NMuMG cells described in *A* were grown in three-dimensional organotypic cultures for 10 days, at which point the percentage of hollowed acini were quantified by phase-contrast microscopy (**, $p < 0.001$; ***, $p < 0.0001$). Representative acini are shown.

tumor bearing animals on a Xenogen IVIS-200 (Xenogen Corporation, Hopkinton, MA); (ii) calculating primary tumor volumes using digital calipers and the equation digital V = $(x^2)(y)(0.5)$, where x is the tumor width and y is tumor length; and (iii) measuring primary tumor weights following their surgical excision on days 21 or 26 post-inoculation. Finally, serial histological sections of control and p130Cas-deficient 4T1 tumors were stained with Ki67 antibodies, and counterstained with hematoxylin as described previously (11). Data were quantified using Image J software. All animal studies were performed in accordance with the animal protocol procedures approved by the Institutional Animal Care and Use Committee of University of Colorado.

Statistical Analysis—Statistical values were defined using an unpaired Student's *t* test, where a p value < 0.05 was considered significant.

RESULTS

Elevated p130Cas Expression Inhibits TGF-β-mediated Smad2/3 Activation—Elevated expression of p130Cas is associated with mammary tumor progression (20), and with the uncoupling of TGF-β to Smad3 activation in epithelial cells

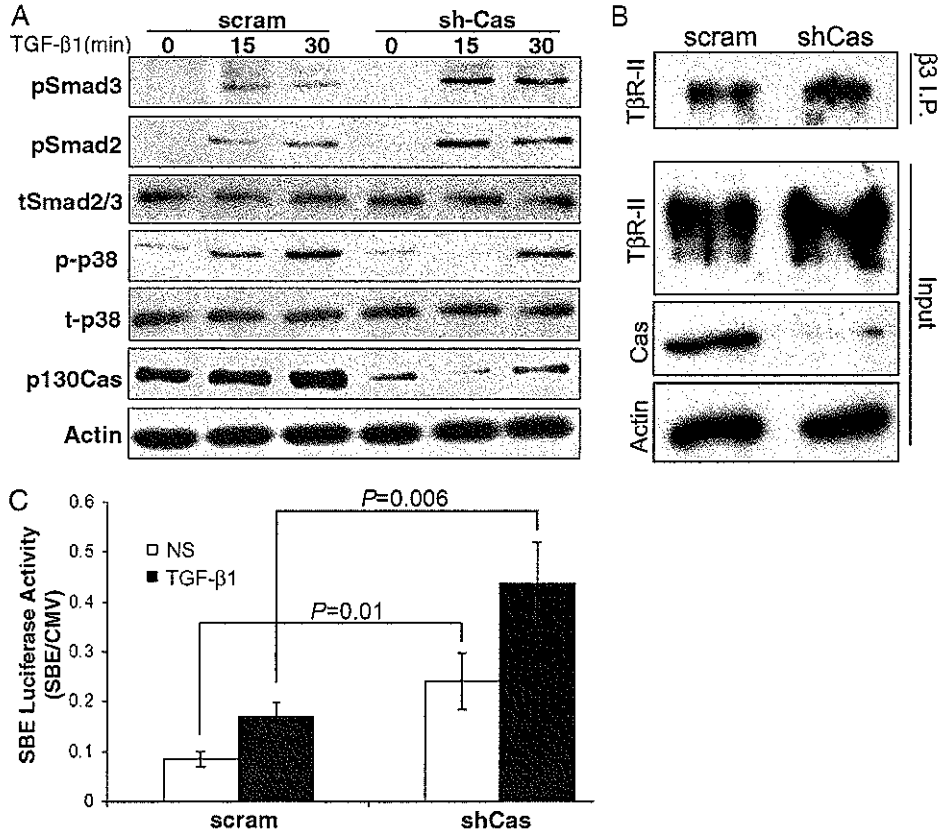

FIGURE 3. **p130Cas deficiency increases Smad2/3 activity in metastatic MECs.** *A*, quiescent 4T1 cells that expressed either a scrambled (*scram*) or p130Cas-specific (*shCas*) shRNA were stimulated with TGF-β1 (5 ng/ml) as indicated, and subsequently immunoblotted with phosphospecific antibodies against Smad3 (*pSmad3*), Smad2 (*pSmad2*), or p38 MAPK (*p-p38*) as shown. Membranes were stripped and reprobed with antibodies against Smad2/3 (*tSmad2/3*), p38 MAPK (*t-p38*), β-actin (*Actin*), and p130Cas as loading controls. *B*, whole cell extracts prepared from control (*scram*) or p130Cas-deficient (*shCas*) 4T1 cells were incubated with β3 integrin antibodies (*β3 I.P.*), and the resulting immunocomplexes were isolated and immunoblotted for TβR-II. Immunoblotting aliquots of the prepared cell extracts (*Input*) served to monitor the levels of TβR-II, p130Cas, and β-actin (*Actin*). Data are representative of three independent experiments and show that p130Cas deficiency does not affect carcinoma-specific formation of β3 integrin·TβR-II complexes. *C*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were transiently transfected with SBE-luciferase (*Renilla*), and subsequently stimulated overnight with TGF-β1 (5 ng/ml). *NS*, no stimulation. Data are the mean ± S.E. ($n = 3$) of *Renilla*/firefly activity ratios.

(21). Unfortunately, the pathophysiological importance of these events, if any, in mediating oncogenic TGF-β signaling in normal and malignant MECs remains unknown. As an initial measure of potential changes in p130Cas expression during mammary tumor progression, we monitored p130Cas protein levels by immunoblotting whole cell extracts prepared from nontransformed human MECs (MCF10A) and their corresponding metastatic derivatives (CA1a) (24–26). As shown in Fig. 1*A*, p130Cas expression was readily increased in human metastatic CAla cells as compared with nontransformed isogenic counterparts. Along these lines, we also observed p130Cas expression to be up-regulated dramatically in the murine 4T1 model of mammary tumor progression (Fig. 1*B*) (26, 27). Indeed, the highly metastatic 4T1 and 66c14 cells expressed significantly more p130Cas than did their moderately metastatic counterparts, 168-Farn and 4T07 (Fig. 1*B*). Consistent with this trend, we found nonmetastatic 67NR cells to express very little p130Cas (Fig. 1*B*). Importantly, the increased expression of p130Cas was consistent with a shift in the balance of TGF-β signaling from primarily that of canonical Smad2 phosphorylation in nonmetastatic 67NR cells to one that included a marked activation of p38 MAPK in metastatic 4T1 and 66c14 cells (Fig. 1*B*). Furthermore, using a measure of the downstream transcriptional activity of Smad2/3, we also observed a drastic diminution in the ability of TGF-β1 to activate Smad2/3 in 4T1 *versus* 67NR cells (Fig. 1*C*). We also monitored changes in the phosphorylation status of p130Cas upon stimulation with TGF-β. As shown in Fig. 1*D*, stimulating 4T1 cells with TGF-β not only readily induced the phosphorylation of Smad2, but also that of p130Cas (Fig. 1*D*). Moreover, the basal levels as well as the ability of TGF-β to induce phosphorylation of p130Cas were abrogated by rendering 4T1 cells deficient in its upstream kinase, FAK (Fig. 1*D*). Interestingly, depletion of FAK elicited a compensatory up-regulation of total p130Cas expression that was consistent with diminished coupling of TGF-β to Smad2 phosphorylation (Fig. 1*D*). Together, these findings are consistent with the notion that p130Cas, irrespective of its phosphorylation status, functions to shift the balance of TGF-β signaling during breast cancer progression by suppressing Smad2/3 activity and supporting p38 MAPK activation in response to TGF-β.

Overexpression of p130Cas Inhibits Smad2/3 Activity and Alters Normal Mammary Epithelial Acinar Formation—To address the role p130Cas in regulating TGF-β signaling, we overexpressed either the full-length protein (Cas-FL) or just the carboxyl terminus of p130Cas (Cas-CT) in normal murine mammary epithelial (NMuMG) cells (Fig. 2*A*). Indeed, overexpression of either Cas-CT or Cas-FL readily decreased the phosphorylation of Smad2 and Smad3 in response to TGF-β1 (Fig. 2*B*). In contrast, TGF-β-induced p38 MAPK phosphorylation was readily increased upon Cas-CT expression, whereas expression of Cas-FL was sufficient to induce the phosphorylation of p38 MAPK even in the absence of added TGF-β (Fig. 2*B*). Furthermore, overexpression of either Cas-CT or Cas-FL also dramatically decreased the extent of basal and TGF-β-induced Smad2/3-dependent transcription (Fig. 2*C*). Functionally, we observed the p130Cas-dependent reduction in Smad2/3 activity to significantly inhibit the cytostatic response of NMuMG cells to TGF-β (Fig. 2*D*). Finally, because TGF-β is critically involved regulating normal mammary gland develop-

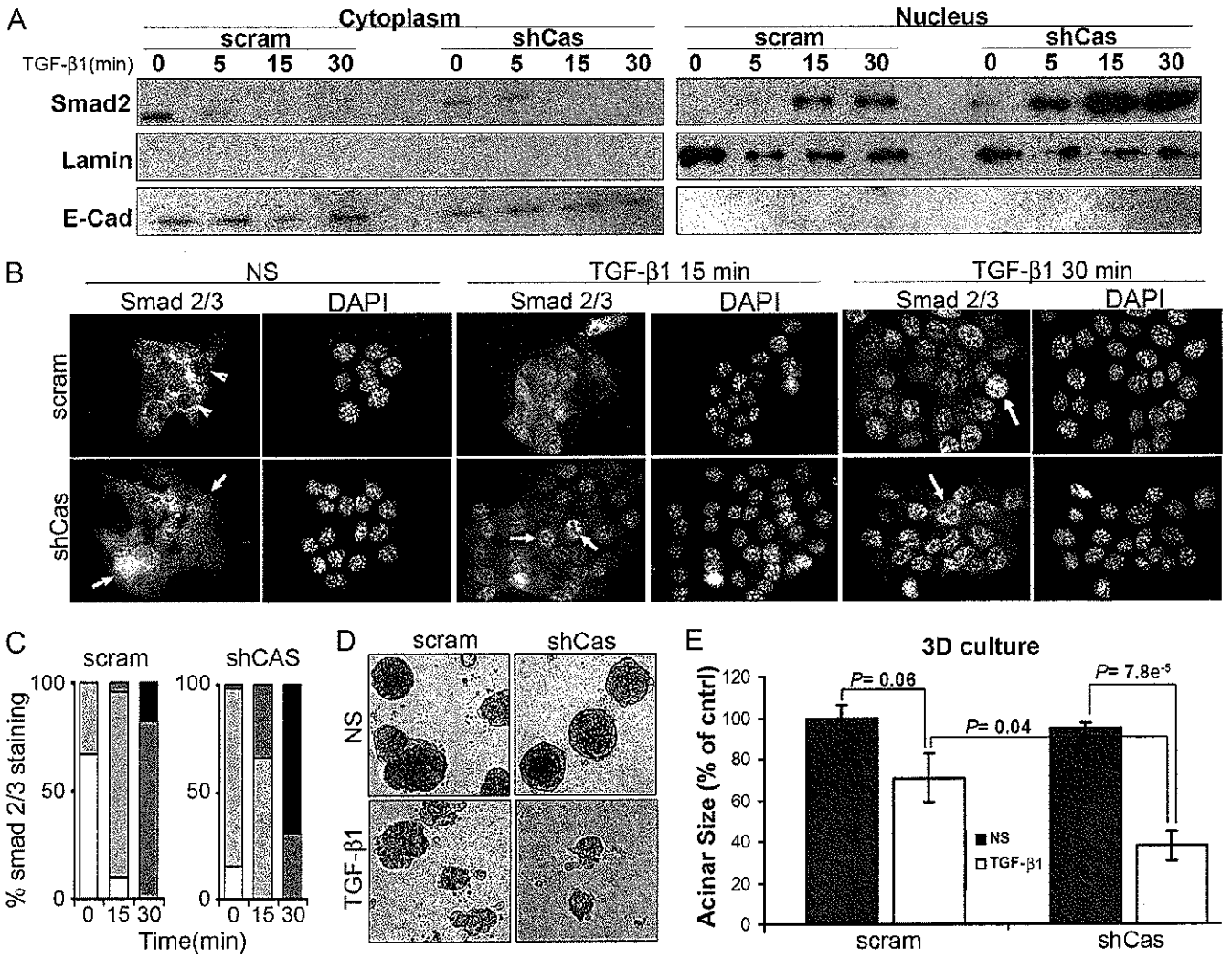

FIGURE 4. **p130Cas deficiency increases Smad2/3 nuclear localization and decreases the proliferation of metastatic MECs.** *A*, quiescent control (*scram*) or p130Cas-deficient (*shCas*) 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for the indicated times, and subsequently lysed, fractionated, and immunoblotted for Smad2/3. Membranes were stripped and reprobed with antibodies against E-cadherin (*E-Cad*) and lamin A/C (*Lamin*) to monitor the integrity of the cytoplasmic and nuclear preparations, respectively. Data are from a representative experiment that was performed three times with similar results. *B*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were stimulated as described in *A* and subsequently fixed and processed for indirect Smad2/3 immunofluorescence, as well as counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*) to visualize cell nuclei. *Arrows* indicate the distinct absence or presence of Smad2/3 in 4T1 cell nuclei. Data are representative images from four independent experiments. *C*, Smad2/3 immunofluorescence data in *B* was quantified as follows: *white bars*, nuclear exclusion; *light gray bars*, cellular diffuse; *dark gray bars*, weak nuclear; *black bars*, strong nuclear staining for Smad2/3. Data are from 10 randomly selected fields for each time point obtained in two independent experiments. *D*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were grown in a three-dimensional organotypic culture for 10 days in the absence or presence of TGF-β1 (5 ng/ml). Representative acini from three independent experiments are shown. *E*, p130Cas depletion restored the cytostatic response of TGF-β in 4T1 cells, which were grown as in *D* prior to quantifying acinar size. Data are the mean ± S.E. of 9 randomly selected fields obtained from three independent experiments.

ment (28–30), we next sought to assess the affect of p130Cas overexpression on the formation of acini by NMuMG cells propagated in a three-dimensional organotypic culture system. Importantly, expression of either Cas-FL or Cas-CT readily invoked acinar filling, a phenotype that recapitulates *in vivo* mammary tumor progression (31). Taken together, these findings clearly indicate that inappropriate up-regulation (see Fig. 1) of p130Cas expression was sufficient to inhibit the physiologic activity of Smad2/3, thereby diminishing the tumor suppressive activities of TGF-β.

p130Cas Deficiency Increases Smad2/3 Activity in Metastatic MECs—Given that elevating p130Cas expression was sufficient to inhibit Smad2/3 signaling stimulated by TGF-β, we next examined how p130Cas deficiency would affect the coupling of Smad2/3 to TGF-β in MECs. To this end, we expressed and screened five independent p130Cas-specific shRNA sequences in NMuMG cells. The general importance of p130Cas in maintaining normal MEC physiology and homeostasis was readily apparent as NMuMG cells that expressed shCas#5 shRNA, which elicited the greatest degree of p130Cas depletion (see supplemental Fig. S1*A*), failed to thrive and survive under extended culture conditions (data not shown). Overall, p130Cas deficiency led to decreased Smad2 expression in NMuMG cells (see supplemental Fig. S1*A*). These findings are consistent with the notion that p130Cas deficiency augments the activity of Smad2/3, which elicits proteosome-directed degradation of Smad2/3 (see supplemental Fig. S1*B*) (23, 32, 33). However, this decrease in Smad2 expression precluded a direct

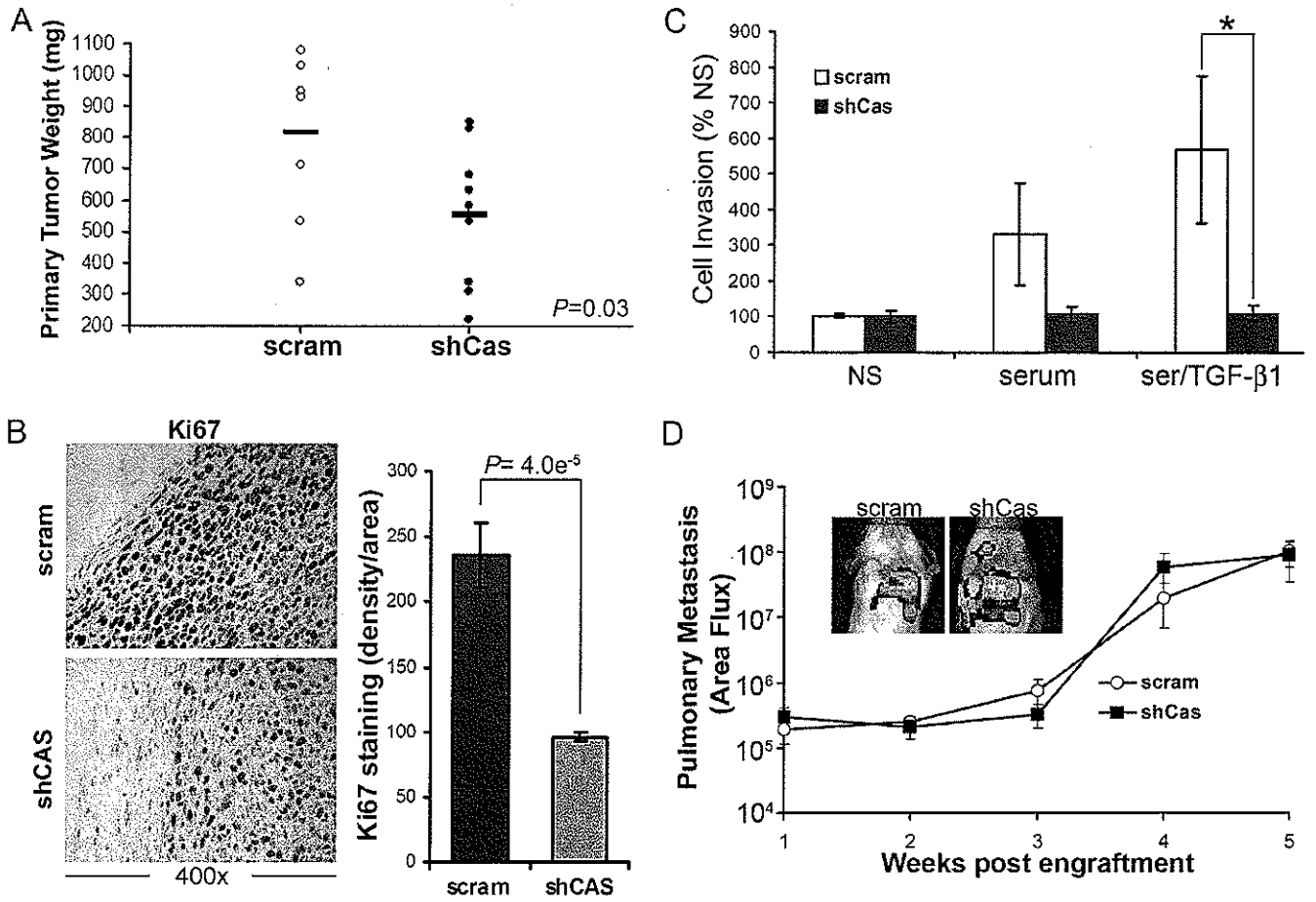

FIGURE 5. **p130Cas deficiency inhibits primary tumor growth and cell invasion.** *A*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were engrafted onto the mammary fat pad of Balb/c mice. Primary tumors were removed surgically 21 days post-engraftment and weighed. *Bar* shows the mean tumor weights for each group (10 mice/group). $p = 0.03$. *B*, histological sections of primary tumors were stained with Ki67 to monitor the proliferative index of control (*scram*) and p130Cas-deficient (*shCas*) 4T1 tumors. Staining intensity was quantified over nine fields of view from three separate tumors/group and showed a decrease in the proliferative index at the invasive front of primary tumors upon p130Cas depletion. *C*, the invasion of control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells through Matrigel was stimulated by 2% fetal bovine serum (*serum*), or by 2% fetal bovine serum supplemented with TGF-β1 (5 ng/ml; *Ser/TGF-β1*). Data are the mean ± S.E. invasion to unstimulated MECs (*NS*) observed in three independent experiments completed in triplicate (*, $p < 0.05$). *D*, 4T1 cell derivatives were engrafted onto the mammary fat pads of female Balb/c mice as described in *A* and pulmonary photon flux readings were determined at the indicated time points post-engraftment. *Inset* shows representative bioluminescent signals of pulmonary metastases 4 weeks post-engraftment.

analysis of the affect p130Cas deficiency elicited on the activation of Smad2/3 by TGF-β in normal MECs. Despite this limitation, aberrant p130Cas expression has been associated with increased breast cancer progression and poorer clinical prognosis (18–20). Therefore, we sought to address the functional impact of p130Cas deficiency on the ability of TGF-β to initiate oncogenic signaling in the 4T1 metastatic model of breast cancer. Indeed, depletion of p130Cas greatly augmented the coupling of TGF-β to Smad2 and Smad3 in metastatic 4T1 cells without affecting total Smad2/3 levels (Fig. 3*A*). We previously demonstrated that the aberrant interaction of β3 integrin with TβR-II in post-epithelial-mesenchymal transition and malignant MECs is critical for the activation of p38 MAPK by TGF-β (11–13). Fig. 3*B* shows that p130Cas is not required for the formation of β3 integrin·TβR-II complexes in 4T1 cells, which serves to explain the slightly diminished coupling of TGF-β to p38 MAPK in p130Cas-depleted 4T1 cells (Fig. 3*A*). Furthermore, p130Cas deficiency not only elicited a significant increase in autocrine-driven SBE-luciferase activity in quiescent 4T1 cells (Fig. 3*C*), but also significantly augmented their induction of this Smad2/3-responsive reporter gene when stimulated by TGF-β (Fig. 3*C*). Accordingly, Smad2/3 localized primarily to the cytoplasm in quiescent parental 4T1 cells, as determined by (i) cellular fractionation coupled to Smad2/3 immunoblotting (Fig. 4*A*), and (ii) indirect Smad2/3 immunofluorescence (Fig. 4, *B* and *C*). In stark contrast, Smad2/3 was present in the cytoplasm and nuclear compartments in quiescent 4T1 cells that lacked p130Cas expression (Fig. 4, *A–C*), a finding consistent with their elevated basal levels of Smad2/3 activity. Moreover, p130Cas deficiency greatly accelerated the rate and extent of Smad2/3 that accumulated in the nucleus following TGF-β stimulation (Fig. 4, *A–C*). Collectively, these findings are consistent with the notion that aberrant p130Cas expression down-regulates the activity of Smad2/3 in metastatic breast cancer cells.

A characteristic phenotype of mammary carcinoma cells, including 4T1 cells, is their resistance to TGF-β-mediated growth arrest when grown in two-dimensional culture systems (12, 34, 35). Interestingly, the resultant increase in Smad2/3 activity elicited by p130Cas deficiency was unable to restore a

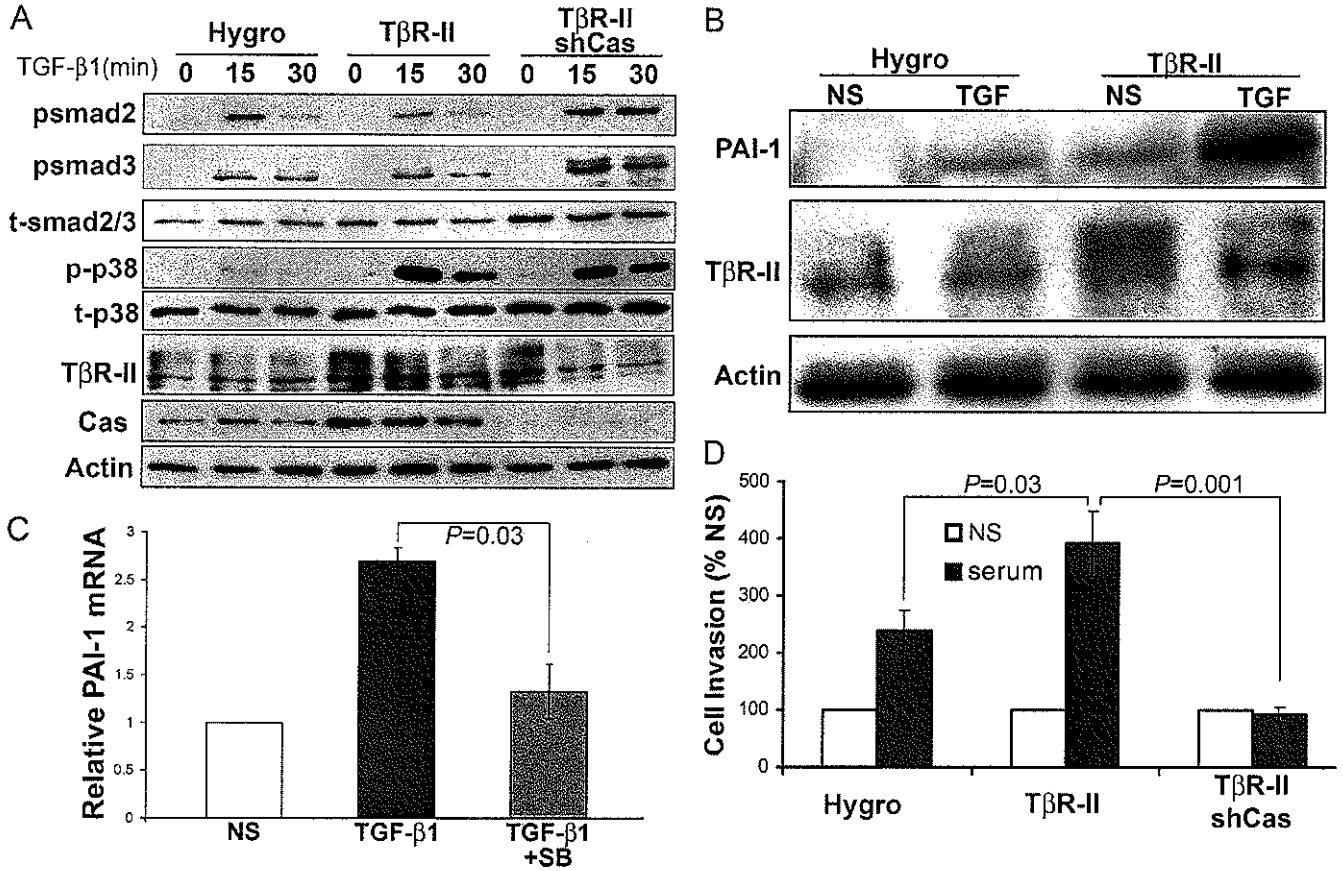

FIGURE 6. **p130Cas balances canonical and noncanonical TGF-β signaling.** *A*, control (*Hygro*), TβR-II- (*TβR-II*), or TβR-II-expressing 4T1 cells deficient in p130Cas expression (*TβR-II/shCas*) were stimulated with TGF-β1 (5 ng/ml) for varying times, and immunoblotted with phospho-specific antibodies against Smad2 (*psmad2*), Smad3 (*psmad3*), and p38 MAPK (*p-p38*) as indicated. Membranes were stripped and reprobed with antibodies against total Smad2/3 (*tSmad2/3*), p38 MAPK (*t-p38*), TβR-II, p130Cas (*Cas*), and β-actin (*Actin*) as loading controls. Data are from a representative experiment that was performed at least three times with similar results. *B*, quiescent control (*Hygro*) and TβR-II-expressing 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h. *NS*, no stimulation. The resulting conditioned medium was collected, precipitated, and immunoblotted for PAI-1. The corresponding cell lysates were probed for TβR-II and β-actin (*Actin*) as loading controls. Data are from a representative experiment that was performed three times with similar results. *C*, 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h in the absence or presence of the p38 MAPK inhibitor, SB208530 (10 μM), and analyzed by semi-quantitative reverse transcription-PCR for PAI-1 mRNA. Data are the mean ± S.E. induction of PAI-1 relative to unstimulated MECs (*NS*) observed in three independent experiments. *D*, 4T1-TβR-II cell variants described *A* were induced to invade synthetic basement membranes by 2% fetal bovine serum (*serum*). Data are the mean ± S.E. invasion relative to unstimulated (*NS*) controls of three independent experiments completed in triplicate.

strong cytostatic response in 4T1 cells upon TGF-β administration (see supplemental Fig. S2*A*). However, it is known that culturing cells on plastic can mask several cell signaling events, most notably those of TGF-β (31, 36, 37). As such, we propagated control and p130Cas-depleted 4T1 cells in three-dimensional organotypic cultures in the absence or presence of TGF-β1 (Fig. 4*D*). In addition to restoring a more rounded, normal acinar structure (Fig. 4*D*), depletion of p130Cas also significantly increased the growth inhibitory effects of TGF-β1 as compared their p130Cas-expressing counterparts (Fig. 4*E*). Taken together, these findings suggest that p130Cas functions to sequester Smad2/3 in the cytoplasm, thereby inhibiting their activity and the cytostatic function of TGF-β. Our findings also suggest that aberrant expression of p130Cas may elicit profound effects on mammary tumor growth regulated by TGF-β.

p130Cas Deficiency Inhibits Primary Mammary Tumor Growth and Cell Invasion—To further assess the role of p130Cas in TGF-β-mediated tumor progression, we engrafted parental and p130Cas-deficient 4T1 cells onto the mammary fat pads of syngeneic Balb/c mice. Indeed, orthotopic tumors lacking p130Cas clearly grew more slowly as compared with their parental counterparts (Fig. 5*A*). Consistent with a reduction in 4T1 tumor weights, we also observed p130Cas deficiency to elicit significantly impaired proliferative indices as determined by Ki67 immunohistochemistry of primary tumor sections (Fig. 5*B*). These data support our *in vitro* findings (Figs. 2*D* and 4*E*) and suggest that measures capable of inactivating p130Cas expression and/or function may provide a novel means to partially restore the tumor suppressive activity of TGF-β.

Through its inclusion in focal adhesion complexes, p130Cas has also been proposed to play a critical role in mediating cell migration and invasion (15). Indeed, p130Cas deficiency abrogated 4T1 cell invasion induced by TGF-β1 (Fig. 5*C*); however, this same cellular condition failed to impact the pulmonary metastasis of 4T1 cells engrafted onto the mammary fat pad of Balb/c mice (Fig. 5*D*). These findings underscore the complexities of carcinoma metastasis *in vivo* and point to the existence of alternative and TGF-β-independent pathways that can compensate for the loss of cellular invasion normally regulated by

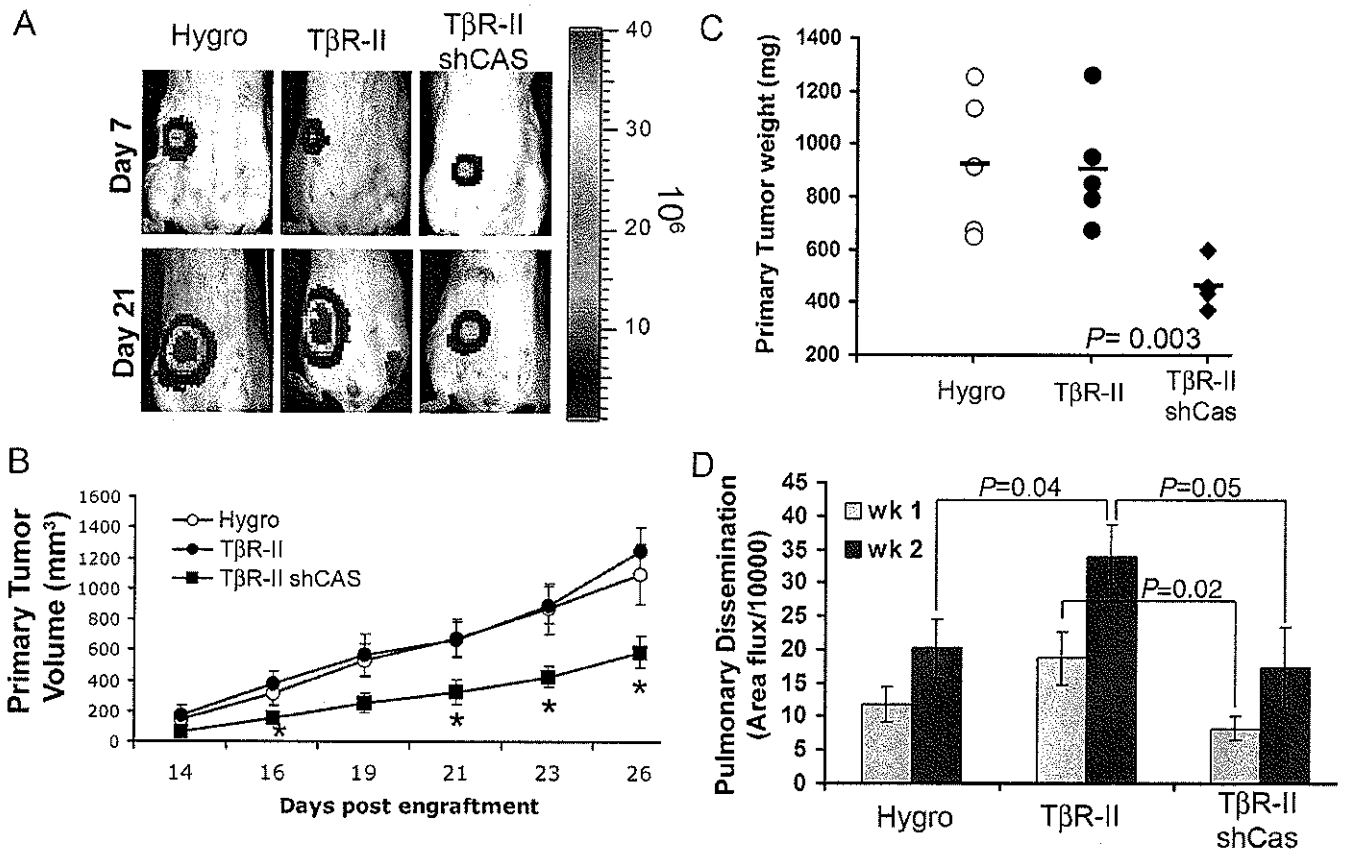

FIGURE 7. **Coupling TβR-II expression with p130Cas deficiency prevents TGF-β-driven breast cancer metastasis.** *A*, control (*Hygro*), TβR-II- (*TβR-II*), and TβR-II-expressing 4T1 cells lacking expression of p130Cas (*TβR-II/shCas*) were engrafted onto the mammary fat pad of Balb/c mice. Bioluminescent visualization of primary 4T1 tumors showed an equal establishment at day 7 post-engraftment, but a significant growth defect at day 26 in TβR-II-expressing 4T1 tumors lacking p130Cas expression. *B*, data are the mean ± S.E. of tumor volumes measured for the indicated 4T1 tumor variants. *, $p < 0.05$, $n = 5$ mice/group. *C*, primary 4T1 tumors were removed surgically 26 days post-engraftment and weighed. *Bar* shows the mean tumor weights for each group (5 mice/group), $p = 0.003$. *D*, data are the mean ± S.E. ($n = 5$ mice/group) of pulmonary photon flux units measured at 1-week intervals following engraftment of the 4T1 variants onto the mammary fat pads.

p130Cas. Indeed, it is tempting to speculate that pulmonary metastasis of p130Cas-deficient 4T1 cells reflects their maintenance of p38 MAPK activity (Fig. 3*A*), a signaling pathway in which we demonstrated previously to be necessary for TGF-β stimulation of 4T1 pulmonary metastasis (11).

p130Cas Balances Canonical and Noncanonical TGF-β Signaling—We previously demonstrated that transgenic expression of human TβR-II in 4T1 cells significantly enhances their invasion (12) and pulmonary metastasis (11) in mice. Therefore, we next sought to utilize this model to specifically address the role of p130Cas in mediating TGF-β-driven tumor progression and metastasis. Indeed, transgenic expression of TβR-II dramatically enhanced the coupling of TGF-β to the activation of p38 MAPK, but had little to no affect on Smad2 or Smad3 phosphorylation (Fig. 6*A*). This shift in TGF-β signaling was reflected by the increased basal and TGF-β-induced expression of the prometastatic factor, plasminogen activator inhibitor-1 (PAI-1; Fig. 6*B*). Moreover, pharmacological inhibition of p38 MAPK activity significantly impaired the ability of 4T1 cells to up-regulate PAI-1 in response to TGF-β1 (Fig. 6*C*). Importantly, rendering these "hyperinvasive" 4T1-TβR-II cells deficient in p130Cas had no appreciable effect on their enhanced ability to phosphorylate p38 MAPK in response to TGF-β; however, this same cellular condition did elicit elevated phosphorylation of both Smad2 and Smad3 (Fig. 6*A*). Thus, diminishing p130Cas expression in 4T1-TβR-II cells restores the physiologic balance between canonical and noncanonical TGF-β signaling (Fig. 6*A*). In accord with their increased p38 MAPK activity and PAI-1 secretion, 4T1-TβR-II cells are significantly more invasive compared with parental 4T1 cells (Fig. 6*C*) (11, 12). Importantly, depleting 4T1-TβR-II cells of p130Cas expression abrogated their enhanced invasiveness mediated by TβR-II expression and a serum stimulation (Fig. 6*C*). Taken together, these findings clearly show that elevated TβR-II expression enhances the coupling of TGF-β to its noncanonical effector, p38 MAPK, leading to augmented PAI-1 expression and cellular invasion. Furthermore, we show for the first time that p130Cas deficiency restores a physiologic balance between canonical and noncanonical TGF-β signaling, and as such, prevents breast cancer cell invasion.

p130Cas Deficiency Prevents Early TGF-β-driven Breast Cancer Dissemination—We next sought to utilize the 4T1-TβR-II model to define the specific role of p130Cas in mediating *in vivo* TGF-β-driven breast cancer progression. Bioluminescent imaging of tumor bearing Balb/c mice showed that parental, TβR-II-, and TβR-II-shCas-expressing 4T1 tumors exhibited similar rates of establishment (Fig. 7*A*, *Day 7*). However, their growth rates thereafter diverged rapidly due to the

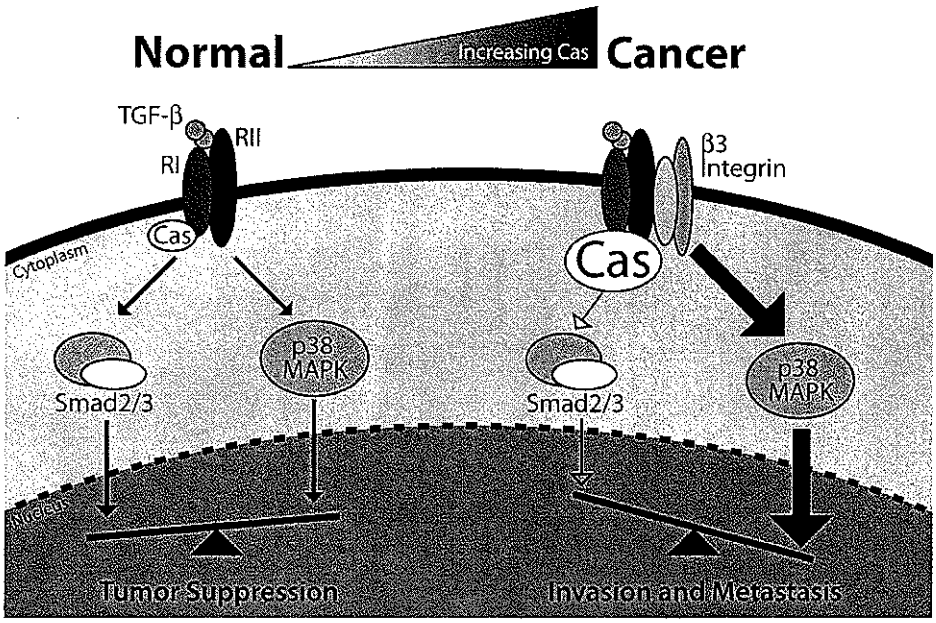

FIGURE 8. **p130Cas functions as a molecular rheostat that maintains the balance between canonical and noncanonical TGF-β signaling.** In normal MECs, physiologic expression levels of integrins and p130Cas maintain the balance between Smad2/3 and p38 MAPK signaling, which collectively support the tumor suppressing and cytostatic functions of TGF-β. During breast cancer progression, aberrant up-regulation of p130Cas expression inhibits Smad2/3 activation in a manner that parallels the inappropriate formation of β3 integrin·TβR-II complexes, which promotes increased coupling of TGF-β to p38 MAPK. Overall, these untoward events shift the balance of TGF-β signaling to favor activation of noncanonical effectors, particularly p38 MAPK, during the acquisition of metastatic phenotypes by breast cancer cells. Importantly, rendering late-stage breast cancer cells deficient in p130Cas enhances the activation of Smad2/3 by TGF-β, which thereby restores its ability to suppress the growth and pulmonary metastasis of breast cancer cells in mice.

inability of TβR-II-shCas tumors to grow out as efficiently as the parental and TβR-II-expressing control cells (Fig. 7, *A* and *B, Day 21*). Importantly, combining TβR-II expression with p130Cas depletion significantly exacerbated (by 10-fold) the growth defects originally observed upon p130Cas depletion in wild-type 4T1 tumors (Fig. 7*C*). Thus, abrogating p130Cas was sufficient in restoring the tumor suppressing activities of TGF-β.

Finally, we found that TβR-II expression elicited a dramatic increase in the early dissemination of 4T1 tumors to lungs as compared with parental cells (Fig. 7*D*). Importantly, this TGF-β/TβR-II-driven metastatic process was specifically inhibited by rendering TβR-II-expressing 4T1 cells deficient in p130Cas (Fig. 7*D*). Taken together, these findings show that p130Cas is critically involved in promoting primary mammary tumor growth, and is specifically required in facilitating early events in TGF-β-driven primary tumor dissemination.

DISCUSSION

TGF-β is a principal player involved in suppressing mammary tumorigenesis, doing so through its ability to maintain the composition of normal MEC microenvironments, and by inhibiting the aberrant proliferation of normal MECs (6, 38). Mammary tumorigenesis has evolved a variety of mechanisms that subvert the tumor suppressing functions of TGF-β, and in doing so, confer oncogenic and metastatic activities upon this multifunctional cytokine (34). Indeed, how TGF-β both suppresses and promotes mammary tumorigenesis remains a fundamental question that directly impacts the ability of science and medicine to effectively target the TGF-β signaling system during the treatment of breast cancer patients. Deciphering this paradox remains the most important question concerning the biological and pathological actions of this multifunctional cytokine (39).

We previously established the importance of aberrant interactions between β3 integrin and TβR-II to promote Src-mediated phosphorylation of TβR-II, which then recruits and binds Grb2. Once bound to phospho-Tyr-284 in TβR-II, Grb2 facilitates TGF-β-mediated activation of noncanonical MAP kinase signaling without affecting the coupling of TGF-β to Smad2/3 (12, 13). Importantly, measures capable of disrupting this signaling axis readily prevent TGF-β from driving breast cancer invasion and metastasis (11, 40). Thus, in addition to establishing the critical importance of p38 MAPK activation in mediating breast cancer metastasis stimulated by TGF-β, these studies also suggested that inappropriate imbalances between canonical and noncanonical TGF-β signaling systems may in fact underlie its prometastatic activities in breast cancer cells. Our findings herein provide the first definitive evidence that (i) canonical and noncanonical signaling imbalances do indeed dictate MEC response to TGF-β, and (ii) p130Cas functions as a novel molecular rheostat that governs the delicate balance between canonical and noncanonical TGF-β effectors. Indeed, overexpression of either full-length or the carboxyl terminus of p130Cas was sufficient to decrease TGF-β-induced Smad2/3 phosphorylation while simultaneously increasing that of p38 MAPK. Moreover, depleting p130Cas significantly increased the activity of Smad2/3 and concomitantly decreased that of p38 MAPK induced by TGF-β, and finally, elevating TβR-II expression amplified the activation of p38 MAPK by TGF-β, which significantly enhanced early metastatic progression of mammary tumors in mice (Fig. 7) (11). In "hypermetastatic" TβR-II-expressing cells, p130Cas deficiency similarly increased the coupling of TGF-β to Smad2/3, an event that negated the proinvasive and prometastatic activities of p38 MAPK in developing 4T1 tumors. Thus, p130Cas functions in balancing the activation status of canonical and noncanonical effectors targeted by TGF-β, findings that are clinically and medically relevant to the development and progression of mammary tumors regulated by TGF-β.

A schematic depicting the function of p130Cas in TGF-β signaling is presented in Fig. 8. Indeed, in normal MECs, TGF-β receptors fail to interact significantly with integrins, which lim-

its TGF-β stimulation of p38 MAPK and the initiation of oncogenic signaling by TGF-β (13, 40–42). The net effect of these signaling events results in tumor suppression by TGF-β. However, during mammary tumorigenesis, p130Cas expression is up-regulated dramatically, as is the aberrant formation of integrin and TGF-β receptor complexes (11–13, 40), which collectively decrease the activity of Smad2/3 and increase that of p38 MAPK and other noncanonical effectors that promote breast cancer metastasis stimulated by TGF-β (11, 40). This signaling imbalance can be potentiated by elevated TβR-II expression and its consequential enhancement of p38 MAPK activation and metastasis (11, 34, 40, 43). In all cases, these various signaling inputs are critically balanced and influenced by the level of p130Cas expression. Indeed, we (see Fig. 1) and others (20) find mammary tumorigenesis to dramatically increase the expression of p130Cas. Based on our findings presented herein, we suggest that this event limits TGF-β stimulation of Smad2/3, which (i) diminishes MEC responsiveness to the cytostatic activities of TGF-β (44); and (ii) promotes amplified coupling of TGF-β to its noncanonical effectors, leading to breast cancer invasion and metastasis. In fact, our findings strongly support the progressive hypothesis that inappropriate imbalances between canonical and noncanonical TGF-β signaling systems underlies the acquisition of metastatic phenotypes in mammary carcinomas, as well as facilitates the oncogenic switch of TGF-β from a tumor suppressor to a prometastatic molecule.

Along these lines, a recent report suggests that murine mammary tumor virus-driven p130Cas expression in mice is sufficient to induce mammary gland hyperplasia (20). However, it was necessary to combine transgenic p130Cas expression with that of HER2 to enhance formation of mammary tumors (20). Although specific effects on TGF-β activity and signaling were not examined in this mouse model, these findings do suggest that the tumor promoting properties of p130Cas only manifest in the face of additional oncogenic signaling inputs (*i.e.* elevated HER2 expression), which mirrors our own results showing that heightened TGF-β signaling (TβR-II expression) requires p130Cas to induce pulmonary dissemination. Moreover, we show that transgenic TβR-II expression led to increased basal and TGF-β-induced production of the prometastatic protein, PAI1, without impacting the phosphorylation of Smad2/3. These findings suggest that (i) p130Cas specifically regulates the activity of Smad2/3 as opposed to that of the TGF-β receptors, and (ii) Smad2/3 expression levels, not those of TGF-β receptors, are rate-limiting during the activation of canonical TGF-β signaling. Thus, p130Cas acts as a molecular rheostat of canonical Smad2/3 and noncanonical p38 MAPK signaling stimulated by TGF-β, and disruption of the balance between these two pathways has dramatic affects on breast cancer growth and progression.

In summary, we demonstrated that p130Cas functions to regulate the balance between TGF-β-mediated activation of Smad2/3 and p38 MAPK in normal and metastatic MECs. Moreover, we provide compelling evidence that p130Cas is both necessary and sufficient to drive the oncogenic activities of TGF-β, including its regulation of mammary tumor growth and the initiation of early steps in the metastatic dissemination of breast cancer cells. Collectively, our findings establish p130Cas as an essential mediator that underlies the oncogenic conversion of TGF-β function, thereby enhancing its ability to promote the progression of mammary carcinomas.

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and dissemination of metastatic cells [13–15,19–22]. Indeed, upregulated LOX expression is essential for hypoxia-induced metastasis of human MDA-MB-231 breast cancer cells in mice [19], and is also observed most frequently in poorly differentiated, high-grade mammary tumors and, consequently, predicts for increased disease recurrence and decreased patient survival [15,19]. Recently, we observed LOX expression to be induced strongly by TGF-β in normal and malignant mammary epithelial cells (MECs), and in mammary tumors produced in mice. Moreover, inhibiting LOX activity or degrading its metabolic byproduct, hydrogen peroxide, antagonizes both the ability of TGF-β to induce the proliferation, epithelial–mesenchymal transition (EMT), and invasion in normal and malignant MECs. Furthermore, we find that LOX antagonism uncouples TGF-β from stimulating Src and p38 MAPK [Taylor M, Schiemann WP. Unpublished Data], whose activities are essential for mediating oncogenic signaling by TGF-β in breast cancer cells [23–25]. Along these lines, future studies need to enhance our understanding of the role of tumor reactive fibroblasts and their production of TGF-β in protecting carcinoma cells from tumoricidal radiotherapies, and also the molecular and cellular mechanisms whereby anti-TGF-β therapies selectively sensitize carcinoma cells, not their adjacent normal counterparts, to ionizing radiation treatments [26–28].

Collectively, these findings highlight the important role that TGF-β plays in governing autocrine and paracrine signaling networks, and more importantly, demonstrate how disrupting the delicate balance between these systems contributes to carcinoma development and progression.

TGF-β & immunosurveillance

In addition to its regulation of stromal fibroblasts, TGF-β present in cell microenvironments also plays an essential role in governing the delicate balance between host immunosurveillance and inflammation, which collectively can determine whether tumor development and progression is induced or inhibited [29,30]. The importance of TGF-β in regulating immune system function and homeostasis is underscored by the findings that:

- TGF-β1-deficient mice exhibit lethal multifocal inflammatory disease [31,32];
- Smad3-deficient mice exhibit defects in the responsiveness and chemotaxis of their neutrophils, and their T and B cells [33];
- Transgenic expression of truncated TβR-II specifically in T cells results in severe autoimmune reactions characterized by multifocal inflammation and autoantibody production [31].

Furthermore, T cell-specific deletion of Smad4 in mice drives T-cell differentiation towards a Th2 phenotype and elevated secretion of IL-4, -5, -6 and -13 [34]. Similar to fibroblasts, the net effect of disrupting paracrine T-cell signaling networks is the development of gastrointestinal carcinomas in these genetically engineered animals [34]. In addition, cancer cells typically increase their production and secretion of TGF-β into tumor microenvironments, as well as into the general circulation of cancer patients [35–37]. Abnormally elevated TGF-β concentrations also are detected within the tumor milieu in response to ECM degradation mediated by resident and recruited leukocytes (i.e., monocytes/macrophages, dendritic cells, granulocytes, mast cells, T cells and natural killer [NK] cells) that either promote or suppress tumor development in a context-specific manner [38].

TGF-β & adaptive immunity

TGF-β suppresses host immunosurveillance by inhibiting the proliferation and differentiation of NK and T cells, and by diminishing their synthesis and secretion of cytotoxic effector molecules, including INF-γ, lymphotoxin-α, perforin/granzyme and Fas ligand [30,39,40]. TGF-β also inhibits the tumor-targeting activities of T and NK cells through its stimulation of Tregs housed within tumor microenvironments [41]. Whereas TGF-β potently inhibits the proliferation of naive $CD8^+$ T cells, this cytokine elicits little-to-no activity in fully differentiated $CD8^+$ T cells owing to their downregulation of TβR-II. Administration of IL-2 or -10 to differentiated $CD8^+$ T cells restores their responsiveness to TGF-β, as does expression of the costimulatory molecule CD28, which promotes the survival of memory/effector phenotypes in thymic and peripheral T-cell populations [30,39,42]. Mechanistically, the immunosuppressive effects of TGF-β transpire in part via Smad3, whose phosphorylation and activation prevents the mitogenesis of $CD8^+$ T cells by:

- Inhibiting their production of IL-2;
- Repressing their expression of c-Myc, cyclin D2 and cyclin E;
- Stimulating the expression of the CDKIs p15, p21 and p27 [30,39,40].

In contrast to its stimulation of cytostasis in $CD8^+$ T cells, TGF-β has no effect on the proliferation of $CD4^+$ T cells, but does inhibit the differentiation of $CD4^+$ T cells into Th1 and Th2 lineages by downregulating T-cell receptor expression, reducing intracellular Ca^{++} signaling and repressing the expression and activation of transcription factors [30,39,40], all of which weaken host immunosurveillance. Collectively, these findings predict that inactivating TGF-β signaling in $CD8^+$ or $CD4^+$ T cells will inhibit tumor formation by elevating host immunosurveillance, a supposition shown to occur during T-cell-mediated eradication of skin [43] and prostate [44] cancers in mice. More recently, TGF-β was observed to promote the development and progression of breast and colon cancers by inducing $CD8^+$ T cells to secrete IL-17, which exerts prosurvival signaling in carcinoma cells [45]. Thus, in addition to improving host immunosurveillance, neutralizing TGF-β function in T cells will also improve tumor resolution by suppressing the activation of carcinoma survival pathways.

TGF-β & innate immunity

In addition to its role in regulating adaptive immunity, TGF-β also plays an essential role in directing activities and behaviors of components of the innate immune system, including NK cells, dendritic cells, mast cells, monocytes and macrophages. Indeed, we defined a novel TAB1:xIAP:TAK1:IKKβ:NF-κB signaling axis that forms aberrantly in breast cancer cells and in normal MECs following their induction of EMT by TGF-β. Once formed, this signaling axis enables oncogenic signaling by TGF-β, in part via activation of NF-κB and its consequential production of proinflammatory cytokines, which promote breast cancer growth in mice in a manner consistent with regulation of innate immunity by TGF-β [46]. Along these lines, TGF-β receptors were observed to associate with those for IL-1β, thereby enabling:

- TGF-β to activate NF-κB
- IL-1β to activate Smad2
- Both pathways to potentiate inflammatory cytokine production [47] and their ability to promote inflammation and the enhanced survival of tumor-associated monocytes [48,49].

In addition, transgenic expression of IL-1β in the stomachs of mice promoted the activation of myeloid-derived suppressor cells (MDSCs) via an IL-1R/NF-κB signaling axis, whose inappropriate and constitutive activation results in the formation of stomach neoplasias [50]. TGF-β is a potent inhibitor of the cytolytic activity of NK cells, presumably by attenuating the activation of their NKp30 and NKD2D receptors, and by inhibiting their production of INF-γ. In addition, TGF-β also represses the activities of dendritic cells by inhibiting their expression of MHC class II, CD40, CD80 and CD86, and TNF-α, IL-12 and CCL5/Rantes [30,39,40,51]. Mast cells are actively recruited to tumor microenvironments by TGF-β where they synthesize and secrete numerous tumor-promoting factors, including histamine, proteases and cytokines (e.g., VEGF and TGF-β) [40,52]. Lastly, TGF-β stimulates monocytes and macrophage chemotaxis to tumor microenvironments, leading to enhanced tumor invasion, angiogenesis and metastasis, and to diminished antigen presentation and immunosurveillance towards developing neoplasms [53,54].

TGF-β & endothelial cells

Angiogenesis is the process whereby new blood vessels sprout and form from preexisting vessels; it also is an essential physiological process that transpires during embryonic development, wound healing and the female reproductive cycle [55,56]. The initiation of pathological angiogenesis has been linked to numerous human diseases, including rheumatoid arthritis, diabetic retinopathy and age-related macular degeneration [56,57]. Interestingly, all solid tumors larger than 1 cm^3 suffer from hypoxia [58] and, as such, initiate angiogenesis as a means of acquiring an efficient supply of nutrients and waste removal, as well as a route for their metastasis to distant locales. Two distinct phases are involved in angiogenesis, namely angiogenic activation and resolution. During the activation phase of angiogenesis, ECs initially exhibit increased vessel permeability and elevated rates of cell proliferation, migration and invasion. In addition, new vessel sprouting is further enhanced by a reduction in EC adhesion, coupled to an alteration in basement membrane integrity. By contrast, angiogenic resolution essentially restores activated ECs to their resting, quiescent phenotypes and promotes the recruitment of perivascular cells that maintain vessel stability and hemodynamics [55–57].

TGF-β plays critical roles in regulating both the activation and resolution phases of angiogenesis [59–62]. Indeed, homozygous deletion of various components of the TGF-β signaling system in mice routinely results in the appearance of vascular and EC defects, particularly in

animals lacking TGF-β1 [63], TβR-I [64], TβR-II [65,66], TβR-III [67,68], Smad1 [69] or Smad5 [70]. In humans, loss or inactivation of endoglin leads to hereditary hemorrhagic telangiectasia type 1 (HHT1) [71,72], while loss of ALK1 results in HHT2 [73,74]. Moreover, the defects associated with HHT1 and HHT2 in humans are phenocopied in knockout mice lacking expression of either endoglin [75,76] or ALK1 [77–79], respectively. Thus, altered expression and/or activity of TGF-β in tumor microenvironments will clearly impact the ability of hypoxic tumors to overcome this impediment to their growth and survival.

Endothelial cells have been reported to express two distinct TβR-Is, namely TβR-I/Alk5 and ALK1. The importance of these two receptors in mediating vessel development by TGF-β is evidenced by the embryonic lethality observed at day E11.5 and E10.5 in mice lacking ALK1 [79] or ALK5 [64], respectively. Recent evidence also suggests that these two type I receptors differentially regulate the coupling of TGF-β to angiogenic activation and resolution. For instance, TβRI/ALK5 activation stimulates Smad2/3 and the subsequent expression of genes operant in mediating vessel maturation, including plasminogen activator inhibitor 1 (PAI-1) and fibronectin [78,80,81]. Moreover, microarray gene expression analyses of EC cells before and after their stimulation with TGF-β confirmed that the activation of a TGF-β:TβR-I/ALK5:Smad2/3 signaling axis does indeed promote angiogenic resolution [61,82]. By contrast, ALK1 activation stimulates Smad1/5 and the subsequent expression of genes operant in mediating angiogenesis activation, including Id1 and IL-1 receptor-like 1 [78,80–82]. Moreover, ALK-1 signaling stimulated by TGF-β requires this cytokine to initially activate TβR-II and ALK-5, which then recruit and activate ALK-1 following its association with TβR-II:ALK-5:TGF-β ternary complexes [78]. Thus, activation of ALK-1 and the induction of angiogenesis by TGF-β must first proceed through its assembly of angiostatic TGF-β receptor complexes (i.e., TβR-II:ALK-5). At present, the molecular mechanisms that initially exclude and then recruit ALK-1 to angiostatic TGF-β receptor complexes remain unknown, but may reflect a delicate balance between TGF-β and other angiogenic factors located within tumor microenvironments. Indeed, low TGF-β concentrations enhance the ability of bFGF and VEGF to stimulate EC proliferation and angiogenic sprouting, while high TGF-β concentrations inhibit these angiogenic activities [62,83]. Along these lines, the pro-angiogenic functions of TGF-β have also been linked to its ability to regulate the expression and/or activities of other angiogenic factors, such as bFGF and VEGF [84]. It is interesting to note that inclusion of TGF-β to Matrigel™ plugs implanted into mice only promotes angiogenesis and vessel development in the presence of bFGF and its ability to create a pro-angiogenic microenvironment [Tian M, Schiemann WP. Unpublished Observation]. Thus, it is plausible that the recruitment of ALK-1 to angiostatic TGF-β receptor complexes may first require the stimulation of accessory angiogenic signals or proteins within activated EC microenvironments. Along these lines, the coupling of TGF-β to angiogenesis is controlled by the presence of endoglin, whose expression is induced by ALK1 and serves to promote EC proliferation, migration and tubulogenesis by antagonizing the activities of TβR-I/ALK5 [60,82].

Collectively, these studies highlight the complexities associated with the ability of TGF-β to regulate EC activities coupled to angiogenesis. Future studies clearly need to:

- Better define the precise mechanisms that enable TGF-β and its downstream effectors to govern the induction of angiogenic or angiostatic gene-expression profiles;
- Establish the impact of EC and perivascular cell differentiation states to influence the angiogenic response to TGF-β;
- Identify the microenvironmental cues and signals that cooperate with TGF-β in mediating angiogenesis activation and resolution.

TGF-β, epithelial–mesenchymal transition & metastasis

The acquisition of invasive and metastatic phenotypes by carcinomas ushers in their transition from indolent to aggressive disease states, during which time immotile, polarized epithelial cells undergo EMT and transdifferentiate into highly motile, apolar fibroblastoid-like cells [85–87]. In doing so, post-EMT carcinoma cells remodel their ECM and microenvironments in a manner that facilitates their intravasation into the vascular or lymphatic systems, as well as their extravasation at distant locales to form micrometastases that ultimately develop into secondary carcinomas [88]. Interestingly, a recent study identified a set of potential metastatic gene signature whose expression is highly associated with the acquisition of pulmonary metastasis by human breast cancers [89]. Included in the metastatic gene signatures are inhibitors of differentiation

(IDs) 1 and 3, which mediate constitutive proliferative signals in newly established pulmonary micrometastases [89]. In addition, the ability of TGF-β to induce angiopoietin-like 4 (ANGPTL4) expression in breast cancer cells enables their retention, extravasation and colonization, specifically in the lungs, not the bone [90]. Pathological reactivation of EMT programs in differentiated cells and tissues not only promotes their invasion and metastasis, but also underlies the development of several human pathologies, such as chronic inflammation, rheumatoid arthritis and chronic fibrotic degenerative disorders, all of which are characterized by dysregulated microenvironmental signaling [85–88,91,92]. In the following sections, we summarize recent developments linking TGF-β to the induction of EMT and metastasis, to the selection and expansion of cancer stem cells, and to the regulation of microRNA expression in developing and progressing neoplasms.

TGF-β signaling & epithelial–mesenchymal transition

Canonical TGF-β effectors & epithelial–mesenchymal transition

The ability of TGF-β to induce EMT and metastasis transpires through the activation of canonical (i.e., Smad2/3-dependent) and noncanonical (i.e., Smad2/3-independent) TGF-β signaling inputs. For instance, inactivating canonical TGF-β signaling in human MCF10ACA1a breast cancer cells by engineering their expression of a dominant-negative Smad3 construct [93] or a TβR-I mutant incapable of activating Smad2/3 (i.e., L45 mutant) [94] significantly reduced their ability to colonize the lung. Along these lines, Smad4-deficiency not only diminished the expression of PTHrP, IL-11 and CTGF in human MDA-MB-231 breast cancer cells, but also abrogated their metastasis to bone in response to TGF-β [95–98]. Interestingly, whereas Smad4-deficiency cooperates with oncogenic K-Ras to induce the initiation and development of pancreatic cancer, the expression and activity of Smad4 is essential for TGF-β stimulation of pancreatic cancer EMT and growth [99]. Similar inactivation of canonical TGF-β signaling by overexpression of Smad7 [100,101] prevents the invasion of breast [102] and head and neck cancers [103,104], as well as the pulmonary metastasis of melanomas [105]. Collectively, these findings highlight the importance of Smad2/3/4 signaling in mediating EMT and metastasis stimulated by TGF-β, and suggest the potential benefit of Smad2/3 antagonists to improve the clinical course of patients with metastatic disease.

Noncanonical TGF-β effectors & epithelial–mesenchymal transition

Noncanonical TGF-β signaling also plays an essential role in mediating TGF-β stimulation of EMT, invasion and metastasis [106]. Included in this growing list of noncanonical effectors targeted by TGF-β are Ras/MAP kinase [107–115], PI3K/AKT [116], Rho/ROCK [117], Jagged/Notch [118], mTOR [119] and Wnt/β-catenin [120]. Collaborative signaling events occurring between NF-κB and oncogenic Ras also mediate EMT and pulmonary extravasation of breast cancer cells in response to TGF-β [121]. Similarly, we identified TGF-β stimulation of NF-κB as an essential pathway operant in coupling TGF-β to the expression of Cox-2, whose activity and production of PGE2 are critical for EMT induced by TGF-β in normal and malignant MECs [122]. Our work [23–25] and research by others [108] has established integrins as key players in mediating EMT, invasion and p38 MAPK activation by TGF-β, as well as its ability to stimulate the growth and pulmonary metastasis of breast cancers in mice [25]. Essential effectors targeted by the formation of integrin:TGF-β receptor signaling complexes are:

- The protein proto-oncogene *Src* and its phosphorylation of TβR-II at Y284, which creates a docking site for Grb2 and ShcA [23–25];
- The adapter molecule Dab2, which facilitates TGF-β stimulation of Smad2/3 and FAK [123,124];
- The protein tyrosine kinase FAK, which coordinates the formation of αvβ3 integrin:TβR-II complexes and, together with its effector p130Cas, is essential for TGF-β stimulation of breast cancer pulmonary metastasis in mice [Wendt MK, Schiemann WP. Unpublished Observation].

In addition, αvβ3 integrin also mediates TGF-β-dependent metastasis of breast cancer cells to bone [125,126]. Collectively, these findings implicate TβR-II as an essential mediator of oncogenic signaling by TGF-β, particularly its ability to promote the acquisition of invasive and metastatic phenotypes at the expense of significantly impacting primary tumor growth [127]. Along these lines, a missense mutation in TβR-II identified in human head and neck carcinomas was observed to promote their EMT and invasion in part via hyperactive protein kinase activity in mutant TβR-II proteins, and also by inappropriate coupling of TGF-β receptors to Smad1/5 activation, as opposed to Smad2/3 [128]. Interestingly,

following its phosphorylation by TβR-II, the tight-junction assembly protein, PAR-6, associates with TβR-I and coordinates the ubiquitination and degradation of RhoA by Smurf1 [129]. The net effect of these TGF-β-dependent events results in the dissolution of epithelial cell tight junctions and the disassembly of their actin cytoskeleton, leading to the induction of EMT.

TGF-β & cancer stem cells

It is important to note that EMT is a normal and essential physiological process that directs tissue development and morphogenesis in the embryo and promotes the healing, remodeling and repair of injured tissues in adults [85–87]. Thus, tumorigenic EMT in many respects reflects the inappropriate reactivation of embryonic and morphologic gene expression programs, and as such, points towards a potential link between EMT and the maintenance of stem cell properties. Accordingly, aggressive and poorly differentiated breast cancer and glioma cells exhibit gene signatures characteristic of stem cells [130], while human and mouse MECs induced to undergo EMT acquire stem cell-like properties, in part via activation of the TGF-β signaling system [131]. Since TGF-β is a master regulator of physiological and pathological EMT [91], these findings suggest that the conversion of TGF-β from a tumor suppressor to a tumor promoter mirrors its ability to induce the selection and expansion of stem cell-like progenitors in post-EMT cells. In fact, TGF-β treatment of malignant, but nonmetastatic human breast cancer cells suppressed their tumorigenicity by diminishing the size of the cancer stem cell pool, and by reducing ID1 expression that results in the differentiation of the progenitor pool [132]. Thus, uncoupling TGF-β from regulation of ID1 expression may dictate whether TGF-β either promotes or suppresses the maintenance and/or expansion of cancer stem cells. Indeed, pharmacological inhibition of TGF-β signaling in cancer stem cells induced an EMT that resulted in their acquisition of a more epithelial-like morphology [133]. Along these lines, future studies clearly need to identify the molecular mechanisms that link TGF-β and EMT to the generation of cancer stem cells, and establish the therapeutic impact of TGF-β in promoting chemoresistance via its stimulation of EMT and the expansion of cancer stem cells.

TGF-β & microRNAs

Finally, accumulating evidence now positions microRNAs as potentially important regulators of the TGF-β paradox. Indeed, expression of miR-21 in breast cancers predicts elevated TGF-β1 expression and a poor clinical prognosis [134], while that in gliomas results in the suppression of multiple components of the TGF-β signaling system, including its ligands (e.g., TGF-βs 1 and 3), receptors (e.g., TβR-II and TβR-III), and effector molecules (e.g., Smad3, Daxx and programmed cell death 4 [PDCD4]) [135,136]. Recently, TGF-β was shown to promote contractile phenotypes in vascular smooth muscle cells by stimulating the processing of primary miR-21 transcripts into their pre-miR-21 counterparts via the formation of Smad2/3:DROSHA complexes. In doing so, cellular levels of miR-21 accumulate rapidly, resulting in diminished expression of PDCD4 and its inability to suppress contractile machinery expression in vascular smooth muscle cells [136]. Similar induction of miR-21 expression took place in Smad4-deficient carcinoma cells, suggesting that TGF-β-regulated miR processing also takes place in epithelial cells in a manner independent of Smad4 [136]. Moreover, miR-21 expression also functions to promote EMT stimulated by TGF-β [137], although the molecular mechanisms underlying this event remain to be determined definitively. In contrast to miR-21 and its role in promoting EMT by TGF-β, microRNA-200 family members and miR-205 function in maintaining epithelial cell polarity and, consequently, in suppressing EMT. Importantly, the ability of TGF-β to induce EMT first requires this cytokine to downregulate microRNA-200 family member and miR-205 expression, which promotes ZEB1 and ZEB2 expression and their initiation of EMT [138]. Thus, aberrant microRNA expression may play a significant role in determining whether epithelial cells sense and respond to the tumor-suppressing functions of TGF-β, or rather to its oncogenic activities.

Conclusions & future perspective

Despite considerable progress over the last decade in defining the molecular mechanisms that underlie the initiation and maintenance of the TGF-β paradox, science and medicine still lack the necessary knowledge and wherewithal to explain and, more importantly, to manipulate the physiopathological actions of TGF-β to improve the clinical course of human malignancies. While it is abundantly clear that TGF-β plays a major role, both directly and indirectly, in regulating the ability of cancer cells to acquire each of the six hallmarks necessary for their malignant progression [139], it

remains unclear as to how these events conspire in regulating the response of developing and progressing neoplasms to TGF-β. For instance, defects in TGF-β function rarely effect primary tumor growth, but more commonly play a significant role in enabling cancer cells to acquire EMT and invasive/metastatic phenotypes. Thus, while it is easy to rationalize why tumors require TGF-β to provide them with a selective EMT and metastatic advantage, teleologically it remains troublesome to assume that these phenotypic changes induced by TGF-β are permanently ingrained in aggressive carcinoma cells. Indeed, cancer cells perpetually locked into a 'vagabond' mentality is counterintuitive to the processes underlying metastasis development and the formation of secondary carcinomas at distant locales. Instead, it appears that the exquisite balance between the functions and behaviors of TGF-β in distinct tissue types become unbalanced and incapable of suppressing disease development, particularly that of neoplastic transformation. Along these lines, the processes underlying the maintenance of normal tissue and cellular homeostasis have been liken to those necessary in facilitating the existence of a well-balanced and harmonious society [140]. The studies highlighted herein are consistent with a role for TGF-β in serving either as a benevolent or corrupt village manager, one whose ultimate agenda is dictated by the prevailing mood of

Executive summary

TGF-β & the tumor microenvironment

Fibroblasts:

- Tumor-reactive stroma plays a critical role in determining whether TGF-β suppresses or promotes tumor formation.
- Loss and/or disruption of paracrine signaling systems between fibroblast and adjacent epithelial cells results in cellular transformation, and in the progression of developing neoplasms.
- Similar inactivation of TGF-β function in epithelial cells also elicits aberrant epithelial:fibroblast paracrine signaling networks that drive malignancy.
- TGF-β stimulation of desmoplastic and fibrotic reactions promotes the formation of stiff, noncompliant microenvironments that select for the expansion of metastatic cells.
- Lysyl oxidase family members are essential for desmoplasia induced by TGF-β, and for stimulating breast cancer metastasis in hypoxic tumor environments.
- Fibrotic reactions enhance TGF-β signaling and may facilitate tumor protection to radiotherapies.

TGF-β & immunosurveillance

- TGF-β is a potent suppressor of inflammation and immune suppression.
- Similar to fibroblasts, altered paracrine signaling by immune cells contributes to tumor formation, particularly in the gastrointestinal track.
- TGF-β is a potent inhibitor of adaptive immunity, which contributes to weaken host immunosurveillance.
- TGF-β also is a potent activator of innate immunity, which contributes to carcinoma progression and metastasis.

TGF-β & endothelial cells

- Angiogenesis is the process whereby new blood vessels develop from pre-existing vessels.
- Angiogenesis also provides cancer cells a route for their metastatic spread.
- Aberrant TGF-β signaling elicits developmental vascular defects that typically result in embryonic lethality.
- Human hereditary hemorrhagic telangiectasia is phenocopied in mice lacking expression of either ALK1 or endoglin.
- TGF-β regulates both the activation and resolution of angiogenesis by differential activation of ALK1 (i.e., pro-angiogenic via Smad1/5/8 activation) or TGF-β receptor (TβR)-I/ALK5 (i.e., anti-angiogenic via Smad2/3 activation).
- Activation of ALK1 by TGF-β requires the presence of ALK5 and TβR-II.

TGF-β, epithelial–mesenchymal transition & metastasis

- Epithelial–mesenchymal transition (EMT) is a process whereby polarized, immotile epithelial cells transdifferentiate into apolar, highly motile, fibroblastoid-like cells.
- TGF-β is a master regulator of normal and tumorigenic EMT.
- EMT is essential for the acquisition of invasive and metastatic phenotypes in carcinoma cells.
- TGF-β induces EMT via stimulation of canonical (i.e., Smad2/3) and noncanonical (i.e., Ras/MAP kinases, PI3K, AKT and Rho/ROCK) pathways.
- β3 integrin, Src and p38 MAPK are essential in facilitating EMT stimulated by TGF-β.
- Aberrant expression of microRNAs in response to TGF-β may drive EMT and metastasis.
- EMT induced by TGF-β may play important roles in generating chemoresistant cancer stem cells.

the village's stromal and microenvironmental constituents. Thus, these findings also underscore and reinforce the need to develop novel pharmacological agents designed to antagonize the oncogenic activities of TGF-β in cancer cells, as well as in their supporting stromal compartments.

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